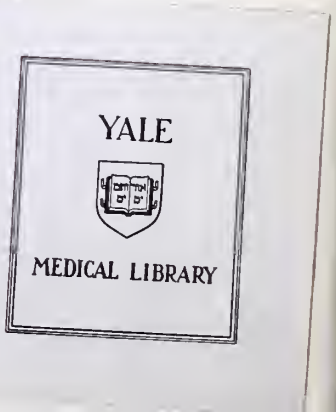
AN INVESTIGATION OF D,L-THREO-N^2-ACETYL-3-FLUOROASPARAGINE METHYL ESTER AS A CHEMOTHERAPEUTIC AGENT

LINDA JEANNE HALL

1978



AN INVESTIGATION OF D,L-THREO-N^2-ACETYL-3-FLUOROASPARAGINE METHYL ESTER AS A CHEMOTHERAPEUTIC AGENT

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A.B., Smith College, 1974

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THESIS ABSTRACT

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D,L-Threo-N^2-acetyl-3-fluoroasparagine methyl ester (AcFAsnMe) was synthesized by Duschinsky at the Swiss Institute for Experimental Research who found that it inhibited L5178Y and L1210 mouse leukemic cell lines. It was initially thought that this compound might be deblocked by acylases and esterases in vivo to yield 3-fluoroasparagine which could act as an asparagine analog to inhibit cell growth. As such it might have a role in the chemotherapy of leukemia, either alone or in conjunction with L-asparaginase. Therefore, in this thesis, attempts were made to remove the blocking groups from AcFAsnMe with several esterases and an acylase. Unfortunately, these enzymes proved ineffective.

To expand the cytotoxicity studies in cell culture systems, a technique was devised to permit assay of drug effects on L5178Y leukemic cell growth in 24-well plastic trays. The cytotoxicity of AcFAsnMe was studied in this system and 50% inhibition of growth was observed at concentrations of 6×10^{-5} M. Addition of up to 20-fold the normal medium concentration of L-asparagine did not significantly protect against the cytotoxic effects of AcFAsnMe.

Therefore, studies with AcFAsnMe were continued from a somewhat different perspective. Duschinsky had shown that an aqueous solution of AcFAsnMe in phosphate buffer pH 7 at room temperature developed a UV maximum at 233 nm within 70 hours. In 0.1 N NaOH this transition to 233 nm absorbing material occurred immediately and was followed by emergence with 15-20 minutes of a maximum at 267 nm. In our studies with L5178Y it was shown that pre-incubation of AcFAsnMe with media for 20 hours prior to addition of cells did not reduce the growth inhibitory effect. This result suggested that one of the degradation products might be responsible for inhibition since at pH 7 AcFAsnMe undergoes complete conversion at 37° to the 233 nm absorbing material within 280 minutes.

An investigation of the chemical nature of the 233 nm absorbing product as well as the other possible degradation products was undertaken. It was shown that at pH 8.8 one mole of acid was liberated for every mole of AcFAsnMe present, with a $T_{\frac{1}{2}}$ of 20 minutes. When the concentration of AcFAsnMe was 0.25 M, conversion to the 233 nm absorbing product by titrimetric addition of 1 N NaOH at pH 8.8 was associated with formation of a crystalline precipitate which was subsequently filtered and recrystallized from H_2O. NMR studies revealed that during conversion of AcFAsnMe to the 233 nm absorbing product, HF had been liberated as well as MeOH. The proposed

structure for the 233 nm absorbing compound was 2-(N-acetyl)-aminomaleimide (NAAM). Further characterization of this compound showed it to be a white crystalline solid which with slow heating, decomposed at >260° and with rapid heating showed evidence of sublimation. From mass spectroscopy studies a molecular weight of 154 was determined, and elemental analysis yielded 46.91% C, 3.83% H, 18.21% N (theoretical - 46.75% C, 3.93% H, 18.18% N). UV spectroscopy and TLC were used to further characterize the degradation products which had formed in addition to NAAM. From these studies, a mechanism for the reaction of AcFAsnMe at pH 8.8 or in culture medium was proposed.

Crystalline NAAM caused approximately 55% inhibition of the growth of L5178Y cells at a concentration of 4×10^{-5} M. Since the other degradation products were not found to be cytotoxic in the same system, it was concluded that NAAM was indeed the compound responsible for inhibition. Presumably, this previously unreported compound acts as an alkylating agent, as numerous other maleimide derivatives have been shown to function. Initial studies with NAAM have shown that it does react with L-cysteine, a strong nucleophile. Further studies are needed to determine the applicability of NAAM in the field of cancer chemotherapy.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my advisor, Dr. Robert Handschumacher, for investing great quantities of time, for his keen insight, and for his continued guidance, support and friendship; to Dr. R. Duschinsky for providing the starting material and therefore the opportunity for this research; to Barbara Stanley for her aid in tissue culture techniques; to Dr. Ian Armitage, Dr. JoAnne Stubbe, Dr. Pauline Chang, and Dr. Ann Jacubowski for their help with the chemical aspects of this thesis; and to Connie Lehman, Dr. Pauline Schwartz and James Moyer for their generous assistance, support and friendship throughout the course of this research.

To My Parents

TABLE OF CONTENTS

INTRODUCTION

INTRODUCTION

The enzyme L-asparaginase is an effective chemotherapeutic agent in the treatment of certain human leukemias (1,2). By converting L-asparagine to L-aspartic acid, it causes death of those tumor cells which require L-asparagine for growth (3,4). Unfortunately, treatment with L-asparaginase is associated with a wide range of toxic effects (5), and the duration of remission in patients responding to the drug is brief (6). For this reason a search for asparagine analogs which might replace or supplement L-asparaginase in the treatment of asparagine-requiring tumors was begun. One such analog, 5-diazo-4-oxo-L-norvaline was shown to inhibit the growth of L5178Y asparagine-dependent leukemic cells (7). Other analogs include the 5-bromo and 5-chloro derivatives of L-2-amino-4-oxopentanoic acid, which at 3×10^{-5} M were shown to inhibit L5178Y cell growth by 48 and 90 per cent, respectively (8). L-2-Amino-4-oxopentanoic acid itself caused 37 per cent inhibition of L5178Y cells (8). In addition to the ability of these asparagine analogs to inhibit the growth of asparagine-dependent cell lines by inhibiting asparagine synthesis, these compounds have provided a means of studying the active site of L-asparaginase, thereby aiding in the development of new asparagine analogs.

Another approach to the chemotherapy of leukemia involves a search for inhibitors of L-asparagine synthetase. This is based on the finding that development of asparaginase-resistant variants from asparaginase-sensitive lines was consistently associated with acquisition of asparagine synthetase activity in both mouse and human leukemias (9,10). Apparently, the resistant cells have derepressed the formation of asparagine synthetase (11). Therefore, if inhibition or prevention of the derepression of this enzyme could be accomplished, the tumor cells should retain their sensitivity to L-asparaginase. In this regard, one study examined the ability of asparagine analogs to inhibit the growth of L5178Y sublines which contain an asparagine-independent form of asparagine synthetase and found the most effective analogs to be L-β-aspartyl methylamide, L-β-aspartyl hydroxamate, and L-β-aspartyl hydrazide (11, 12). When L-β-aspartyl methylamide was administered to mice bearing various asparaginase-resistant tumors, life span was increased from 31 to 79 per cent.

Unfortunately, there has been little success using the known inhibitors to re-establish sensitivity in cases which have developed clinical resistance. Possible contributory reasons include (13): (a) dietary L-asparagine may be carried to the tumor cell in compartments which are impermeable to L-asparaginase; (b) L-asparagine may be synthesized by alternative routes not susceptible to the known inhibitors; (c) many different L-asparagine synthetases may exist with

different susceptibility to inhibition; and (d) more powerful inhibitors of L-asparagine synthetase may be required to completely inhibit the synthesis of L-asparagine. In this regard, a systematic examination was made of a wide variety of compounds as inhibitors of L-asparagine synthetase of L-asparaginase resistant L5178Y leukemia (13). The nitrosoureas BCNU, CCNU, and methyl-CCNU, as well as showdomycin, for example, were found to inhibit L-asparagine synthetase at 1mM concentrations in addition to possessing antitumor activity. Unfortunately, the correlation of enzyme inhibitory potency with oncolytic potency could not be established.

Thus, the search for asparagine analogs which may expand the spectrum of L-asparaginase therapy continues. A fluorinated analog of L-asparagine would seem to be a logical candidate based on the high reactivity and known toxicity of compounds such as 5-fluorouracil (14). β-β-Difluoroasparagine has just recently been synthesized, but was found to be inactive in non-toxic doses when tested <u>in vivo</u> in CDF-1 mice with asparagine-dependent L5178Y leukemia (15). Although several mono-fluorinated amino acids such as β-fluoroalanine (16), α-fluoro-β-alanine (17), and 5-fluoronorvaline (18) have been prepared, synthesis of β-fluoroasparagine has never been reported. This is most likely due to the relative instability of certain fluorinated amino acids due to loss of the fluorine atom by β-elimination as hydrofluoric acid (19). In this regard, the attempted synthesis of β-fluoroaspartic acid by routine methods was unsuccessful (20).

This thesis presents an investigation of the recently synthesized mono-fluorinated asparagine analog, D,L-threo-N^2-acetyl-3-fluoroasparagine methyl ester (AcFAsnMe) (21), which has been kindly supplied by Dr. Robert Duschinsky at the Swiss Institute for Experimental Research. It was hoped that <u>in vivo</u> this compound might undergo deacylation and demethylation thus yielding 3-fluoroasparagine. Preliminary studies of AcFAsnMe by Duschinsky indicated that it caused dose-dependent growth inhibition of L5178Y and L1210 mouse leukemia cells in cell culture (22). In addition, L-asparagine appeared to provide some protection against the cytotoxicity, suggesting that the drug was acting as an asparagine analog. The drug is stable in aqueous solution at pH 4 to 6 but at pH 7.6, it decomposes completely in less than 5 hours as measured by the appearance of an ultraviolet absorption maximum at 233 nm. Therefore, the question was raised as to whether AcFAsnMe itself or the degradation product was responsible for inhibition of cell growth.

Thus, in this thesis, the cytotoxicity of AcFAsnMe against L5178Y mouse leukemia cells with and without L-asparagine was further investigated. The susceptibility of this blocked analog of L-asparagine to esterases and acylases was also studied to determine if 3-fluoroasparagine was liberated from the parent compound. Finally, the biologically active degradation product formed from AcFAsnMe was chemically characterized to be 2-(N-acetyl)-aminomaleimide (NAAM).

A mechanism for the mode of its formation from AcFAsnMe is presented along with proposals concerning the nature of the inactive degradation products.

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Cell Culture Studies with D,L-Threo-N^2-acetyl-3-fluoro-asparagine Methyl Ester (AcFAsnMe).

All lymphoblasts used in these experiments were obtained from stock cultures of L5178Y mouse leukemia cell lines which were maintained between 1 x 10^4 and 1 x 10^5 cells/ml by serial passage in Fischer's medium with 10% horse serum (23), both from Grand Island Biological Company. An 8.0 mM solution of L-asparagine (Calbiochem) was prepared in Fischer's medium, sterile filtered and supplemented to contain 10% horse serum. This concentration included the 0.4 mM L-asparagine present in Fischer's medium. A 5.2 mM solution of L-glutamine (Nutritional Biochemical Corporation) was prepared in a similar manner and added to some cell cultures. This concentration included the 1.4 mM L-glutamine already present in Fischer's medium.

AcFAsnMe was kindly supplied by Dr. R. Duschinsky of the Swiss Institute for Experimental Research, Lausanne. Stock solutions (2.4 x 10^{-4} M) were prepared in distilled water and sterile filtered. The pH of such solutions was approximately 5 and assured stability prior to addition to the cultures. Subsequent dilutions as required were made with sterile water. All additions of drug were in less than 10% of the culture medium.

Preliminary studies of AcFAsnMe by Duschinsky (22) indicated that it is stable at its acidic pH, showing only

end absorption in the ultraviolet spectrophotometer. However, in phosphate buffer pH 7, 25°, the solution develops a UV maximum at 233 nm (ε = 8400, based on the original concentration of AcFAsnMe) within 70 hours (half-life = approximately 19 hours). At 37°, a 10^{-4} M solution of the compound in 10^{-2} M phosphate buffer pH 7.65 decayed completely within 280 minutes, measured by formation of the maximum at 233 nm. In 0.1 N NaOH, at 25°, formation of an immediate temporary maximum that occurred at 230-235 nm was followed by emergence of a maximum at 267 nm (ε = 8800, based on the original concentration of AcFAsnMe) within 15 to 20 minutes.

Cultures of L5178Y cells were grown in Linbro 24-well plates with 1.0 ml final volume per well. Compounds were added in 0.1 ml to 0.8 ml Fischer's medium containing 10% serum with or without added L-asparagine. One tenth ml of L5178Y cell suspension was added to start the cultures at approximately 10^4 cells/ml. The cultures were incubated at 37° in a 5% CO_2-in-air humidified incubator.

In some experiments AcFAsnMe was pre-incubated in media with or without L-asparagine for 20 hours prior to addition of cells. Growth in these solutions was compared with freshly prepared solutions of AcFAsnMe in media with or without L-asparagine.

Cell culture plates were examined under light microscopy for signs of contamination and/or cell damage at 24, 48, and 72 hours. To assess the rate of growth, the contents of each well were removed by Pasteur pipet into 9 ml

of 0.9% NaCl and cell counts were determined by use of the Coulter Counter. All cell growth data points are the average of triplicate determinations. The doubling rates for L5178Y controls using this culture technique compared favorably with those found using standard cell culture tubes containing 5 ml of media (see Discussion).

II. Enzymatic Treatment of AcFAsnMe.

Pseudocholinesterase or acylcholine acyl-hydrolase (Sigma) (1 unit hydrolyzes 1.0 μmole of butyrylcholine per minute at pH 8.0, 37°) was dissolved in 0.01 M KH_2PO_4 buffer pH 7.5, and 3.6 to 7.8 units were added to various substrates to start the reaction. Esterase type I from hog liver (Sigma), a suspension in 3.2 M $(NH_4)_2SO_4$ solution (1 unit hydrolyzes 1.0 μmole of ethyl butyrate per minute, pH 8.0, 25°) was diluted in buffer and between 5.0 and 6.5 units were added to the various substrates to start the reaction. Esterase type II or carboxylic ester hydrolase from hog liver (Sigma), a suspension in 3.2 M $(NH_4)_2SO_4$ solution, pH 6.0 (1 unit hydrolyzes 1.0 μmole ethyl butyrate per minute at pH 8.0, 25°) was diluted in buffer and 7.0 to 14.0 units were added to test substrates.

A 100,000g supernatant from a mouse liver homogenate was used as a source of non-specific esterases (24). Four CD-1 mice 8 to 10 weeks old were killed by cervical dislocation. A 20% weight per volume homogenate of the livers was prepared with a Potter-Elvehjem homogenizer in 0.25 M sucrose (pH 7.5) and centrifuged at 100,000g for one hour at 3°. The

supernatant was added to the various reaction mixtures in amounts equal to 5 and 20% of the total volume of the reaction mixture.

The activity of the above enzymes on the following substrates was tested: succinylcholine chloride (Sigma), propionylcholine chloride (Calbiochem), tri-fluoro-N-acetyl methyl ester of aspartic acid (TFAMeAsp) (kindly supplied by Dr. Pauline Chang of the Department of Pharmacology) and AcFAsnMe.

The liberation of acid was monitored as the ester was cleaved according to the procedure of Augustinsson with a Radiometer pH meter and titrator unit (25). Between 2.0 and 4.0 ml of solution containing 20 to 80 μmoles of substrate were placed in a small test tube in which the pH electrode and titrant pipet were immersed. The reaction mixture was stirred magnetically and rapidly adjusted to between pH 7.0 and 8.0 with a solution of NaOH (standardized to 1.000 N). Several minutes of titration were performed in the absence of enzyme to assess non-enzymatic hydrolysis of the substrate. The enzyme was added and the rate of acid liberation determined for at least 10 minutes.

In all calculations, the acid produced by spontaneous liberation from substrate or enzyme alone was subtracted from the acid liberated during the reaction in the presence of substrate and enzyme. The enzyme activity was expressed in μmoles of substrate hydrolyzed per minute per unit enzyme.

Hog kidney acylase (Sigma) with a deacylation activity of 800 μmoles/hour/mg with N-acetyl-L-methionine as substrate was dissolved in 0.01 M potassium phosphate buffer, pH 7.0. The test substrates N-acetyl-L-asparagine (Sigma) and AcFAsnMe were dissolved in the same buffer.

Initially, 1.0 ml N-acetyl-L-asparagine (1 mM) or AcFAsnMe (2 mM) solution was incubated at 25° with 0.25 mg acylase and samples were removed for analysis at 5, 10, and 15 minutes. In the second set of experiments conducted at 37°, substrate concentrations were increased to 20 mM and the amount of acylase increased to 2.5 or 5.0 mg per ml.

Acylase activity was monitored by a fluorometric assay based on the reaction of the liberated ∝-amino group with o-phthalaldehyde to produce a fluorescent compound. One tenth ml portions of the reaction mixture were added to 3.0 ml of buffered reagent which was prepared fresh daily according to the method of Roth (26). To 90 ml of 0.05 M borate buffer, pH 8.0, 50 mg of o-phthalaldehyde (Durrum) in 1.0 ml 95% ethanol and 10 μl of 2-mercaptoethanol were added and mixed. Fluorescence was monitored with a Farrand MK-1 spectrofluorometer with the monochromaters set at λ_{ex} = 340 nm and λ_{fl} = 455 nm and values were recorded in microamperes after a one minute reaction time (27). A standard curve was prepared with L-asparagine in the range 2.5 to 16 μM (Figure 1). For calculation, the amount of fluorescence obtained from the presence of ∝-amino groups in the enzyme alone and from any non-enzymatic hydrolysis of substrate was subtracted from the

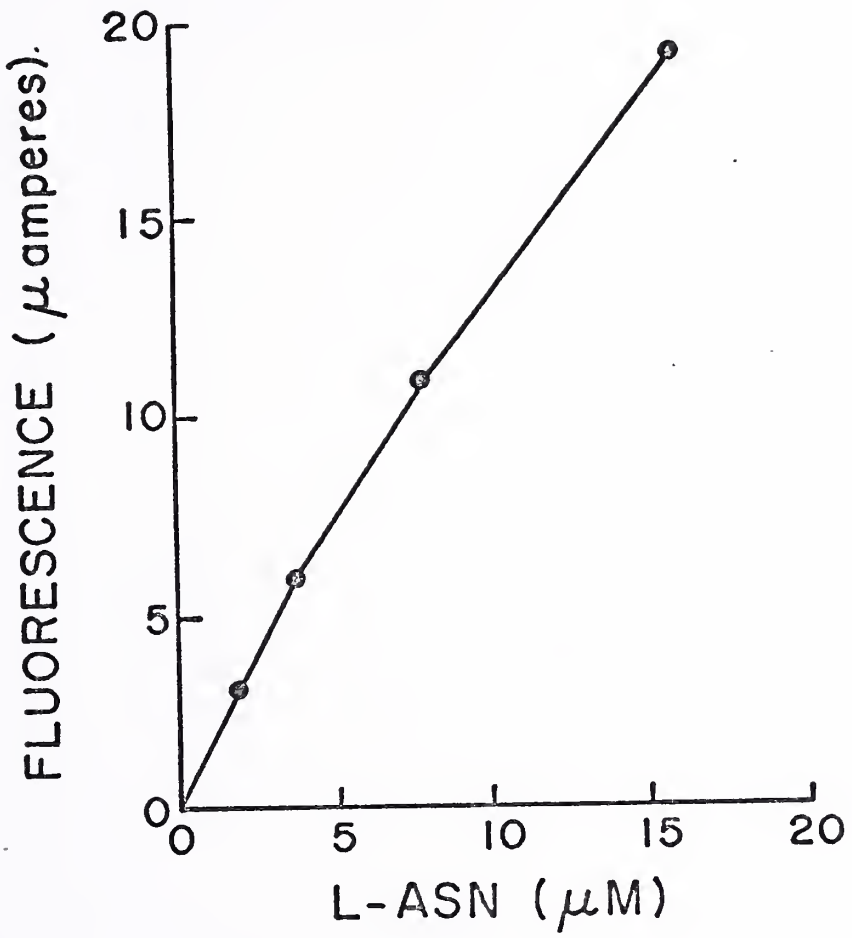


Figure 1. Standard Curve for the Detection of L-Asparagine (L-Asn) with o-Phthalaldehyde. One-tenth ml L-Asn in distilled water (0.06 to 0.5 mM) was added to 3.0 ml buffered reagent and fluorescence was recorded after 1 minute. Details are given in the text.

fluorescence obtained during the reaction of acylase and substrate. Enzyme activity was expressed in μmoles of substrate deacylated per minute per mg enzyme.

III. Chemical Studies on the Formation of 2-(N-Acetyl)-aminomaleimide (NAAM).

Ultraviolet spectra were determined with a Cary 15 spectrophotometer. Nuclear magnetic resonance spectra of hydrogen were determined by Dr. Ann Jakubowski with a Varian T-60 analytical NMR spectrometer at 60 MgHz. Structural groups were assigned on the basis of the charged integrals and the chemical shifts relative to the external standard tetramethylsilane (TMS) = 0.00 ppm (28). Fluorine resonance spectra were performed by Dr. Ian Armitage with a Bruker HFX 90 NMR spectrometer modified for multinuclear operation, in Fourier transformed mode at 84.6 MgHz for ^{19}fluorine.

Mass spectroscopy data was determined at Wesleyan University through arrangements made by Dr. Ann Jakubowski, and elemental analysis was performed by Baron Analytical Services, Orange, Ct.

Thin-layer chromatography was performed on 20 x 20 cm silica gel sheets (Baker Chemical Company). The solvent contained n-butanol, acetic acid and water in a 4:1:1 ratio, respectively. Compounds were detected under an ultraviolet lamp. In the case of AcFAsnMe it was necessary to heat the plate to 100^{o} for 3 to 5 minutes to visualize a UV absorbing spot.

RESULTS

RESULTS

I. Cell Culture Studies with D.L-Threo-N^2-acetyl-3-fluoro-asparagine Methyl Ester (AcFAsnMe).

The cytotoxicity of AcFAsnMe against L5178Y cells is shown in Figure 2. At 2.4×10^{-4} M AcFAsnMe, nearly total cell lysis was observed microscopically even though Coulter counts indicated stasis of growth, whereas at the lower concentrations dose-dependent growth inhibition was seen. L-Asparagine was added in an attempt to prevent the growth inhibition caused by AcFAsnMe; although a minimal protective effect was observed, this concentration of L-asparagine also caused a slight increase in the growth rate of controls (Figure 3).

Since it had been shown that AcFAsnMe was unstable in culture, these same concentrations of drug were pre-incubated for 20 hours in the absence of cells (Figure 4). The decomposition products formed under these conditions also caused a similar degree of growth inhibition.

Additional cell culture studies were done to determine whether the minimal protective effect seen by L-asparagine during incubation of L5178Y cells with AcFAsnMe could be reproduced by substitution of another amino acid. L-Glutamine was found to provide an equivalent protective effect (Figure 5). Thus, the specificity of AcFAsnMe as an asparagine analog might be questioned.

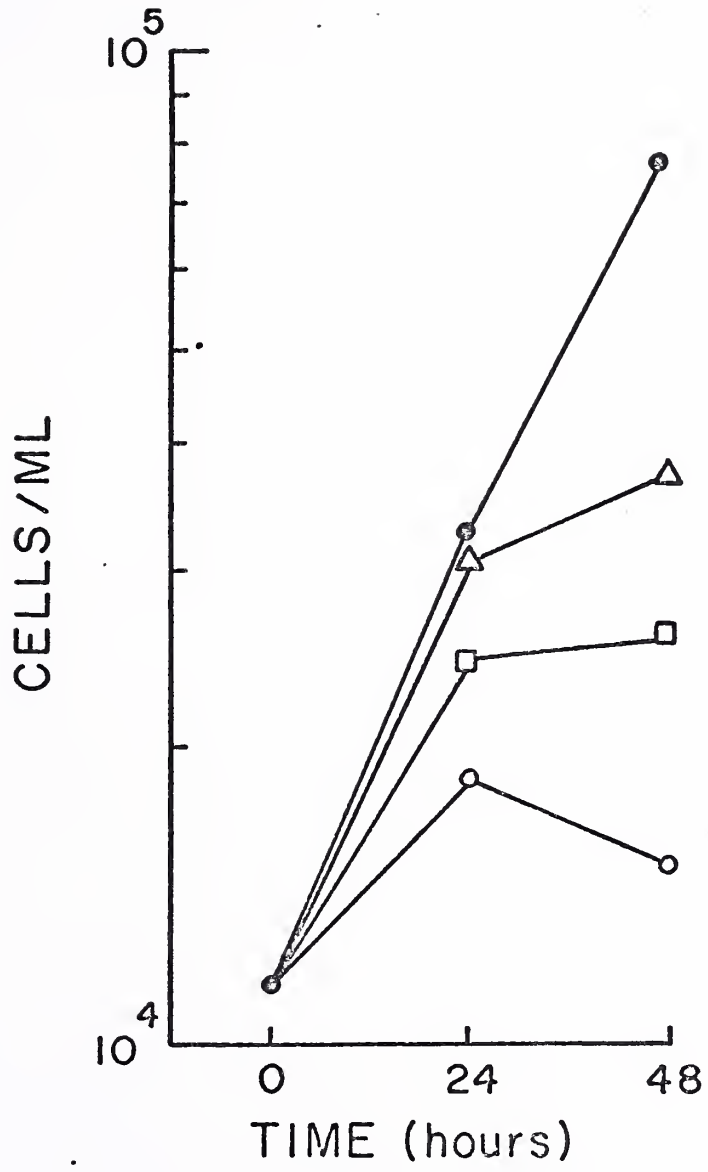


Figure 2. The Growth Inhibition of L5178Y Cells by AcFAsnMe.

- ●——● = Control
- △——△ = 6.1×10^{-5} M AcFAsnMe
- □——□ = 1.2×10^{-4} M AcFAsnMe
- ○——○ = 2.4×10^{-4} M AcFAsnMe

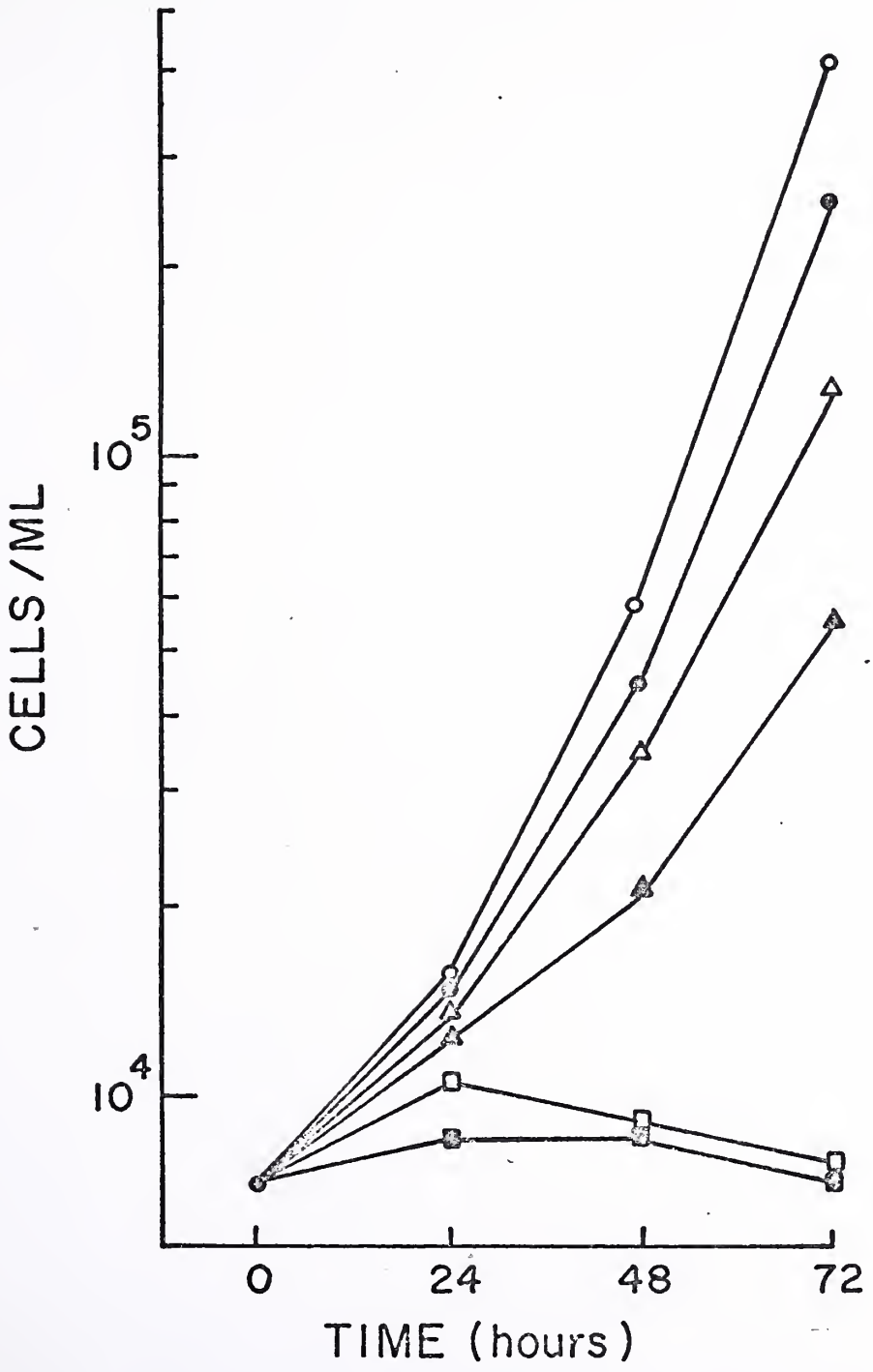


Figure 3. The Effects of AcFAsnMe and L-Asparagine (L-Asn) on Growth of L5178Y Cells.
●——● = Control
○——○ = Control with 8.0×10^{-3} M L-Asn
▲——▲ = 6.1×10^{-5} M AcFAsnMe
△——△ = 6.1×10^{-5} M AcFAsnMe with 8.0×10^{-3} M L-Asn
■——■ = 2.4×10^{-4} M AcFAsnMe
□——□ = 2.4×10^{-4} M AcFAsnMe with 8.0×10^{-3} M L-Asn

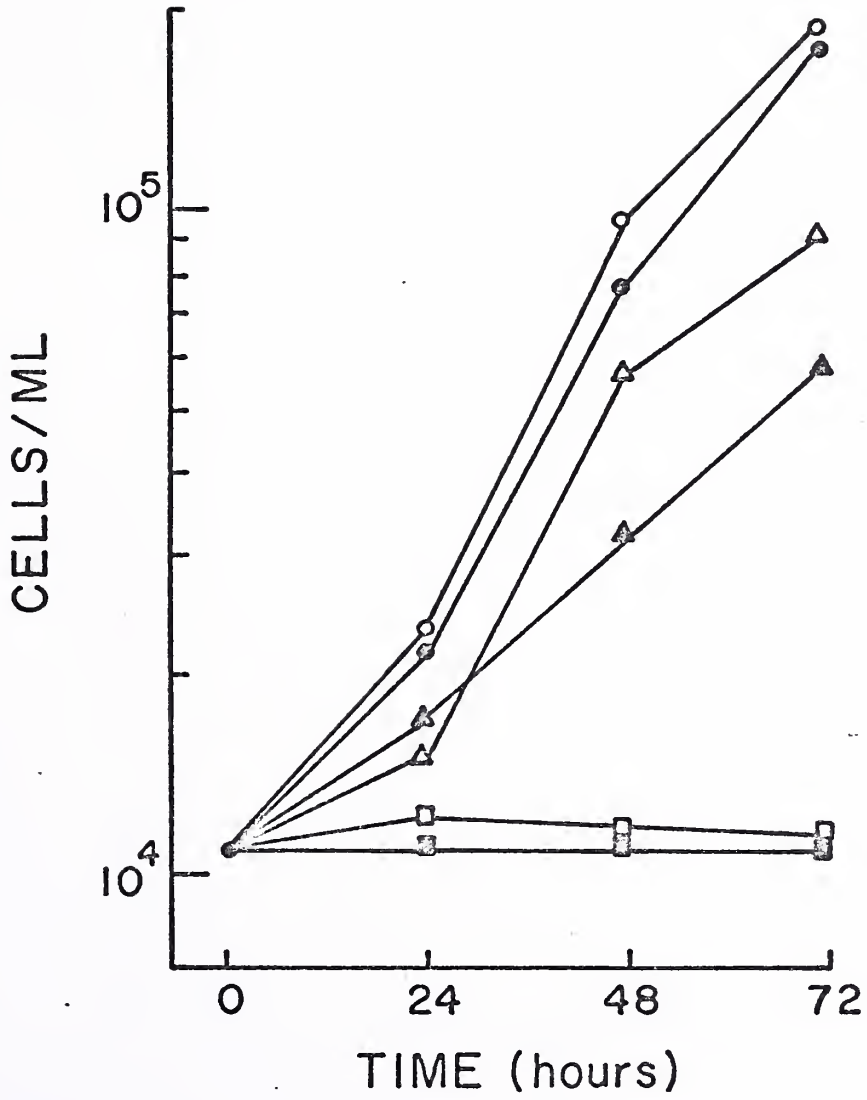


Figure 4. The Effects of AcFAsnMe and L-Asparagine (L-Asn) on Growth of L5178Y Cells. AcFAsnMe was pre-incubated for 20 hours in media with or without L-Asn prior to addition of cells.

●——● = Control
○——○ = Control with 8.0×10^{-3} M L-Asn
▲——▲ = 6.1×10^{-5} M AcFAsnMe
△——△ = 6.1×10^{-5} M AcFAsnMe with 8.0×10^{-3} M L-Asn
■——■ = 2.4×10^{-4} M AcFAsnMe
□——□ = 2.4×10^{-4} M AcFAsnMe with 8.0×10^{-3} M L-Asn

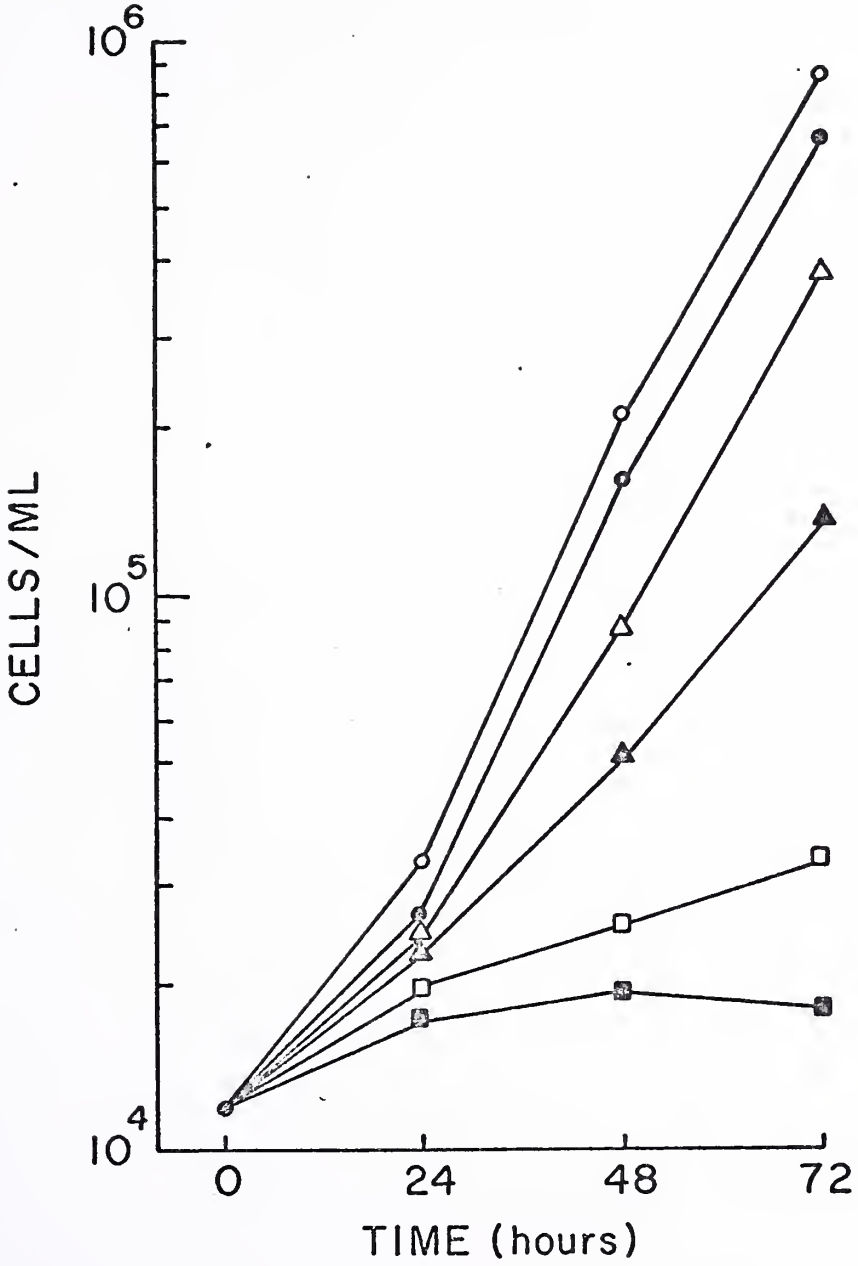


Figure 5. The Effects of AcFAsnMe and L-Glutamine (L-Gln) on Growth of L5178Y Cells.

●——● = Control
○——○ = Control with 5.2×10^{-3} M L-Gln
▲——▲ = 6.1×10^{-5} M AcFAsnMe
△——△ = 6.1×10^{-5} M AcFAsnMe with 5.2×10^{-3} M L-Gln
■——■ = 1.2×10^{-4} M AcFAsnMe
□——□ = 1.2×10^{-4} M AcFAsnMe with 5.2×10^{-3} M L-Gln

II. Enzymatic Treatment of AcFAsnMe.

An attempt was made to remove enzymatically the blocking groups from AcFAsnMe to liberate free 3-fluoroasparagine which was initially proposed as the compound responsible for inhibition. Table I gives the hydrolysis rates for the various test substrates by the enzymes. Although succinylcholine and propionylcholine chloride acted as substrates for esterase type I and pseudocholinesterase, respectively, the tri-fluoro-N-acetyl methyl ester of L-aspartic acid (TFAMeAsp), a compound similar in structure to AcFAsnMe, and AcFAsnMe itself were not hydrolyzed to any significant degree by either of these enzymes. However, a 20 per cent by volume supernatant (100,000g) from mouse liver homogenate containing non-specific esterases was found to hydrolyze both TFAMeAsp and AcFAsnMe as evidenced by liberation of acid at a low but significant rate (Table 2).

AcFAsnMe was also incubated with hog kidney acylase I in an attempt to remove the acyl group. The amount of α-amino group liberated during incubation of substrate and enzyme after correction for non-enzymatic hydrolysis of substrate and α-amino groups present in the enzyme itself was within the linear range of the standard curve (see Figure 1, Materials and Methods). N-Acetyl-L-asparagine was deacylated at a rate of 0.74 µmoles per minute per mg enzyme at 25°, but there was no evidence of deacylation of AcFAsnMe. Even at 37° with a 10-fold increase in substrate and enzyme concentrations, the observed rate of AcFAsnMe deacylated was less than 0.20 µmoles

TABLE I

Substrate	Enzyme	Hydrolysis Rate (μmoles hydrolyzed/ min/unit enzyme, 25°)
Succinylcholine chloride	Esterase, Type I	0.20
Succinylcholine chloride	Esterase, Type II	0.044
Propionylcholine chloride	Pseudocholinesterase	0.20
TFAMeAsp	Pseudocholinesterase	<0.004
TFAMeAsp	Esterase, Type I	<0.015
TFAMeAsp	Esterase, Type II	<0.010
AcFAsnMe	Pseudocholinesterase	<0.004
AcFAsnMe	Esterase, Type I	<0.006
AcFAsnMe	Esterase, Type II	<0.005

Table I. The Hydrolysis of AcFAsnMe and Other Substrates by Various Esterases. All substrates were present at a concentration of 10 mM. Control rates indicating non-enzymatic hydrolysis of substrate were <0.001. Details of the experimental technique as well as the exact units of enzyme added to start the reaction are given in Materials and Methods.

TABLE II

Substrate	Supernatant (% volume)	Hydrolysis Rate (μmoles hydrolyzed/ minute, 25°)
Propionylcholine chloride	5	<0.01
Propionylcholine chloride	20	0.04
TFAMeAsp	5	0.07
TFAMeAsp	20	0.30
AcFAsnMe	5	<0.02
AcFAsnMe	20	0.13

Table II. The Hydrolysis of AcFAsnMe and Other Substrates by Supernatant (100,000g) from Mouse Liver Homogenate. All substrates were present at a concentration of 20 mM. There was no apparent liberation of acid from enzyme alone and control rates for substrate alone were 0.002. Details of the experimental technique are given in Materials and Methods.

per minute per mg enzyme. Further studies would be needed to determine whether AcFAsnMe is indeed hydrolyzed by this acylase to a significant degree.

III. Chemical Studies on the Formation of 2-(N-Acetyl)-aminomaleimide (NAAM).

Duschinsky reported that incubation of AcFAsnMe in culture media gave rise to an increased absorbance at 233 nm. Since essentially equal inhibition of L5178Y cell growth was observed after 20 hour pre-incubation, investigation of the chemical nature of the 233 nm absorbing product was undertaken. A solution of AcFAsnMe (2.5 ml, 1 x 10^{-2} M) in distilled water was rapidly titrated to an endpoint of pH 8.8 with a Radiometer pH meter and titrator. The subsequent liberation of acid was monitored at pH 8.8 by the addition of 1.000 N NaOH until the reaction was complete (Figure 6). One mole of acid was liberated for every mole of AcFAsnMe present. The initial rate of acid production at pH 8.8 was 0.7 μmoles per minute. To monitor the change in UV absorbance during this base-catalyzed reaction, AcFAsnMe (10^{-4} M) was prepared in 0.05 M borate buffer pH 8.8 and scanned at 10 minute intervals between 210 and 300 nm in the UV spectrophotometer to monitor the formation of the 233 nm absorbing product as well as other changes in UV absorbance (Figure 7). It should be noted that the presence of buffer ion may effect the reaction of AcFAsnMe at pH 8.8 and therefore the reaction of this compound in distilled water may be different from that in buffer. At any

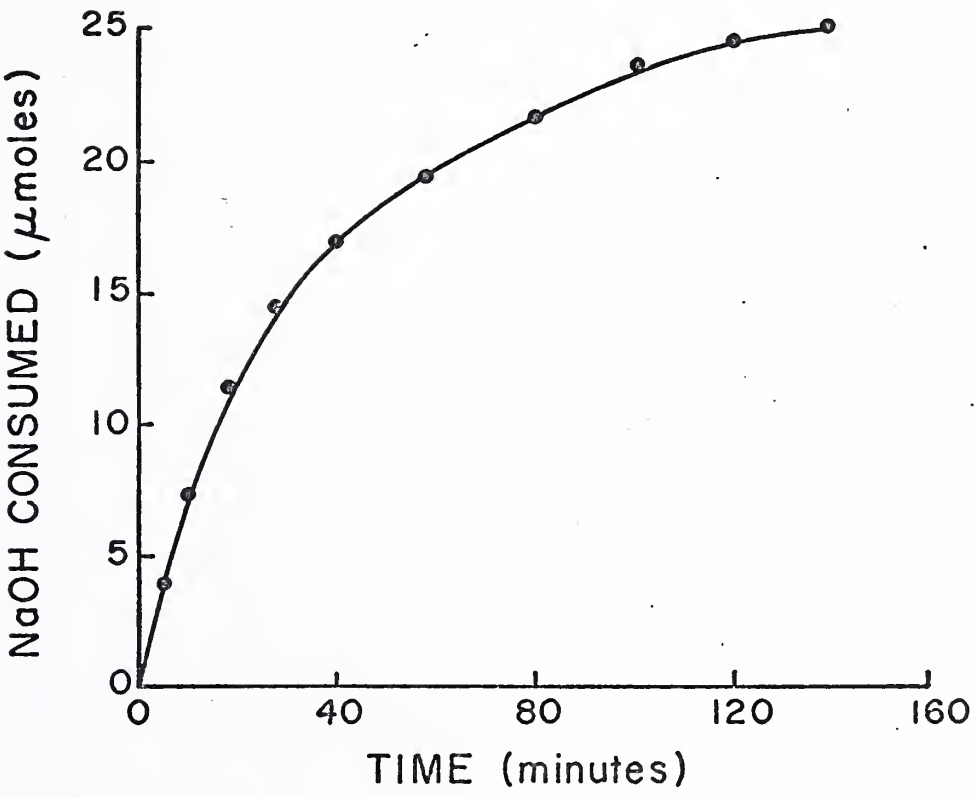


Figure 6. The Liberation of Acid from AcFAsnMe at pH 8.8. AcFAsnMe was present at an initial concentration of 0.01 M with 25 μmoles available for reaction. Details of the experimental technique are given in the text.

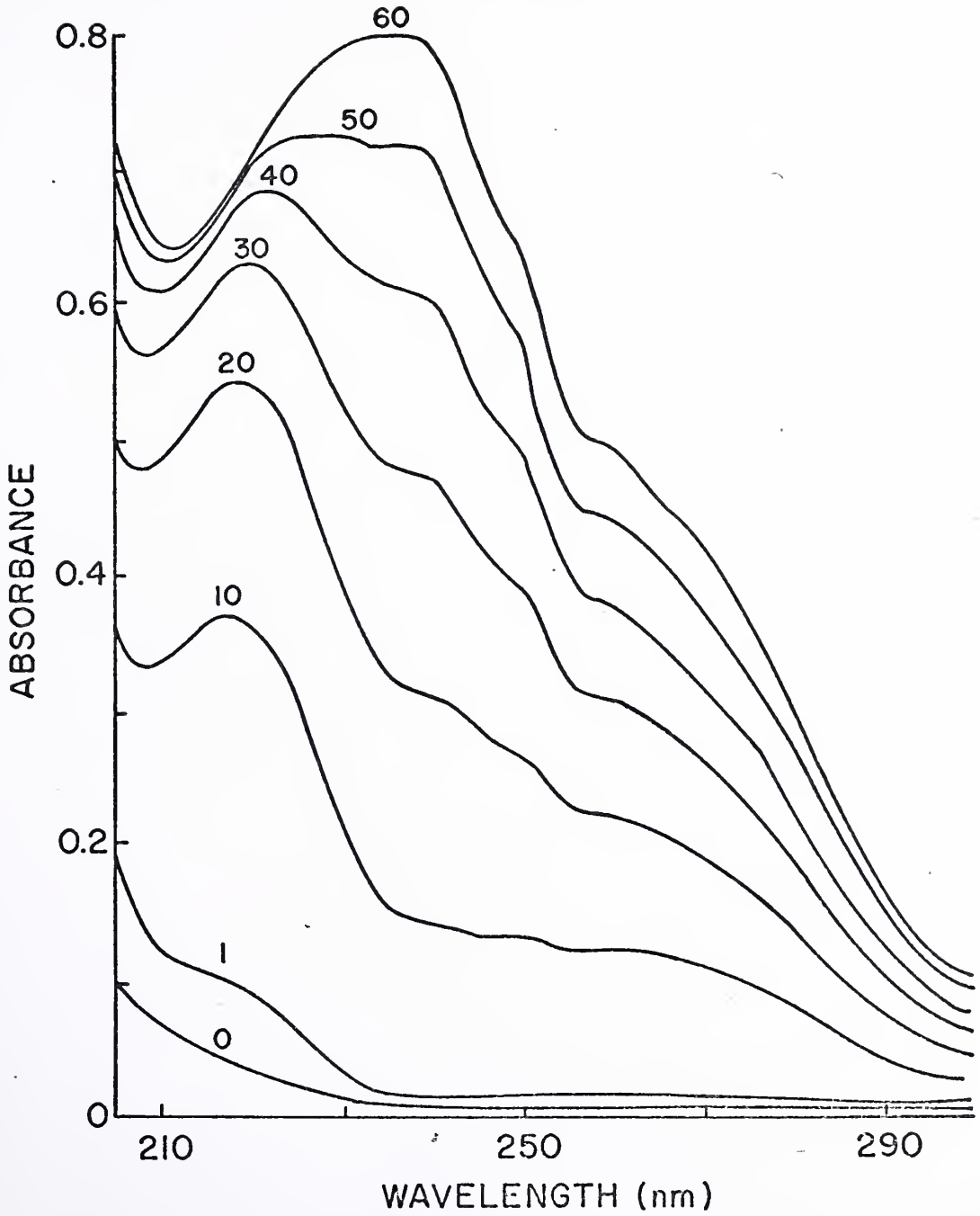


Figure 7. Ultraviolet Spectra During the Liberation of Acid from AcFAsnMe at pH 8.8. AcFAsnMe was present at a concentration of 10^{-4} M in 0.05 M borate buffer pH 8.8. The number on each spectrum indicates the reaction time in minutes. Details are given in the text.

rate, it appears from Figure 7 that the formation of 233 nm absorbing product is preceded by that of a substance which absorbs at lower wavelengths. Along with the formation of a peak at 233 nm, the spectra slso indicate the simultaneous formation of at least two other products. Certainly, it is apparent that the reaction of AcFAsnMe at pH 8.8 is not a simple one. The liberation of acid from AcFAsnMe was also monitored at pH 10.0 by the previously described method. This resulted in a more than 5-fold increase in the rate of acid production. However, the increase from pH 8.8 to pH 10.0 also resulted in a greater than 10-fold increase in the rate of formation of absorbance at 267 nm (calculated from ε = 8800 which is based on the original concentration of AcFAsnMe) and therefore subsequent titrations were performed at the lower pH to optimize the formation of the 233 nm product.

When the starting concentration of AcFAsnMe was increased to 0.25 M (50 mg in 1.0 ml water), the conversion to 233 nm absorbing product was accompanied first by a color change of the solution from clear to faint yellow. Within minutes a crystalline precipitate formed which was filtered under suction to yield 20.5 mg; recrystallization from water gave 14.5 mg. The ultraviolet spectrum of the recrystallized product in water showed a UV maximum at 233 nm (ε = 17,800) with a broad peak in the range 315-330 nm (Figure 8). There was very little absorbance in the 267 nm range. The NMR spectrum of the recrystallized product in deuterated dimethyl sulfoxide (DMSO) gave the following chemical shifts relative to the

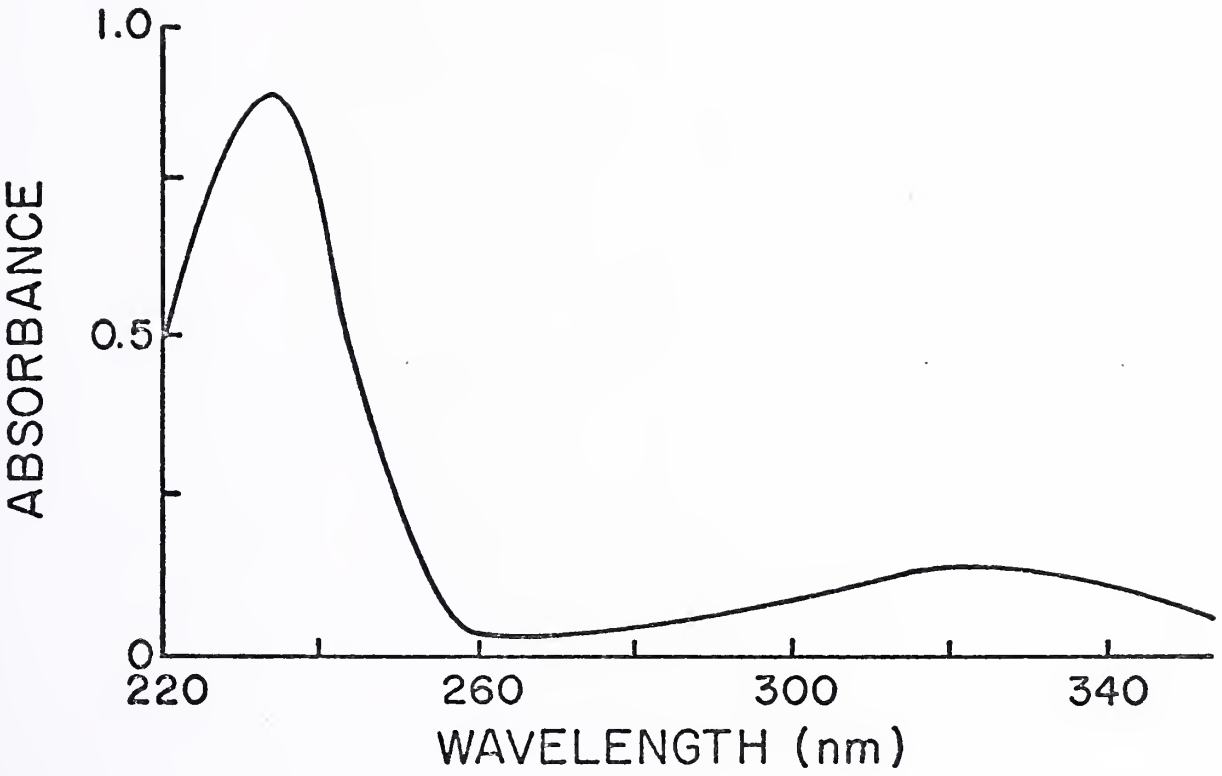
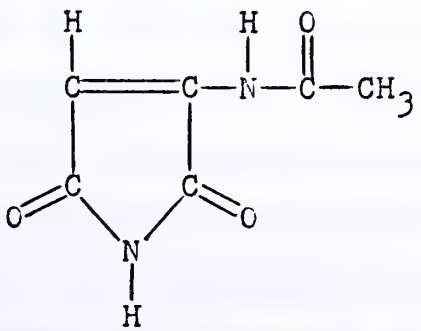


Figure 8. Ultraviolet Spectrum of Recrystallized NAAM. The compound was present at a concentration of 5.0×10^{-5} M in distilled water.

external standard tetramethylsilane (TMS): 2.0 ppm (3H, singlet), 6.1 ppm (1H, singlet). This is to be compared with the NMR spectrum of AcFAsnMe in deuterated water (D_2O) with the same standard: 2.2 ppm (3H, singlet), 3.9 ppm (3H, singlet), 5.3 ppm (1H, doublet of doublets, $J_{CHCHF} = 34$ Hz, $J_{CHCH} = <2$ Hz), 5.7 ppm (1H, doublet of doublets, $J_{FCH} = 46$ Hz, $J_{CHCH} = <2$ Hz). It was thus evident that formation of 233 nm absorbing product from AcFAsnMe had resulted in liberation of MeOH as well as HF. This was in agreement with the fluorine magnetic resonance studies which showed the chemical shift of the fluorine present in AcFAsnMe in D_2O to be 122 ppm upfield relative to the external standard tri-fluoroacetate. This peak was not present in a spectrum of the recrystallized 233 nm absorbing product, again suggesting that fluorine had been liberated during the reaction of AcFAsnMe at pH 8.8. A fluorine resonance spectrum did reveal the presence of fluoride ion in the filtrate after removal of the 233 nm absorbing material.

From the above data, the proposed structure for the 233 nm absorbing compound is 2-(N-acetyl)-aminomaleimide (NAAM):



It is a white crystalline solid which is stable for months when dessicated at 3°. It is sparingly soluble in cold

water and very soluble in DMSO. With slow heating it decomposes at 260° and with rapid heating there is evidence of sublimation. Mass spectroscopy showed the parent ion to be at m/e 154 with a molecular (parent) ion at m/e 155. Thus, the molecular weight of NAAM was determined to be 154 (29). Elemental analysis yielded 46.91% C, 3.83% H, 18.21% N (theoretical - 46.75% C, 3.93% H, 18.18% N).

Thin-layer chromatography with n-BuOH:AcOH:H_2O (4:1:1) as solvent revealed NAAM to be a strongly fluorescent compound (R_f = 0.66), whereas AcFAsnMe in the same solvent was detected under UV light only after heating at 100° for several minutes (R_f = 0.52) as described by Duschinsky.

Investigation of the UV absorbing materials present in the aqueous filtrate after removal of NAAM (Figure 9) was then undertaken. The filtrate contained considerable material that absorbed in the region of 265 nm. To determine whether this product had formed from NAAM, a 3.6×10^{-2} M aqueous solution of NAAM was adjusted to pH 9.6 and the consumption of base monitored at this pH with the Radiometer pH meter and titrator for 60 minutes. At this time, the pH was inadvertently readjusted to pH 10.0, which explains the increase in rate observed at this point (Figure 10). The reaction was complete after 110 minutes at which time nearly one mole of acid had been liberated (30 μmoles) for every mole of NAAM (36 μmoles). At completion of the reaction, an ultraviolet spectrum showed the formation of a single peak (Figure 11) (ε = 10,400, based on the original concentration

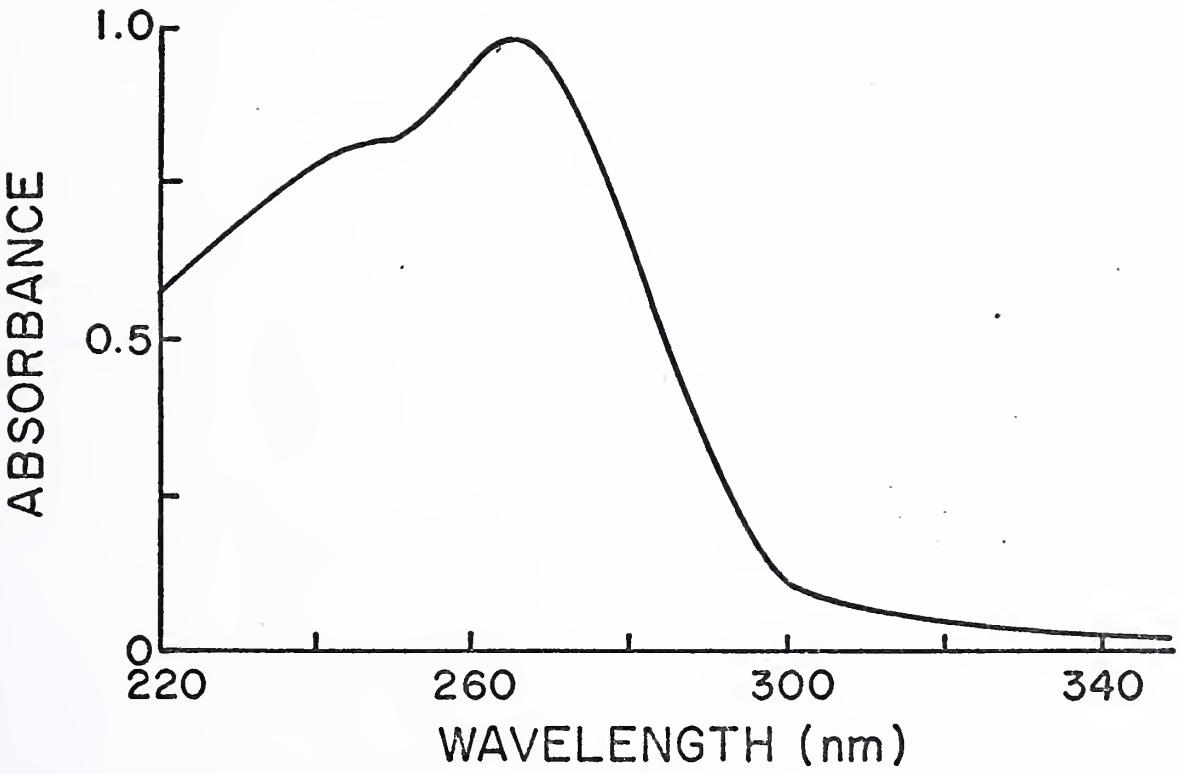


Figure 9. Ultraviolet Spectrum of Filtrate After Removal of NAAM. The initial concentration of AcFAsnMe was 0.36 M. The reaction occurred at pH 8.8 until complete, at which time NAAM was removed by filtration.

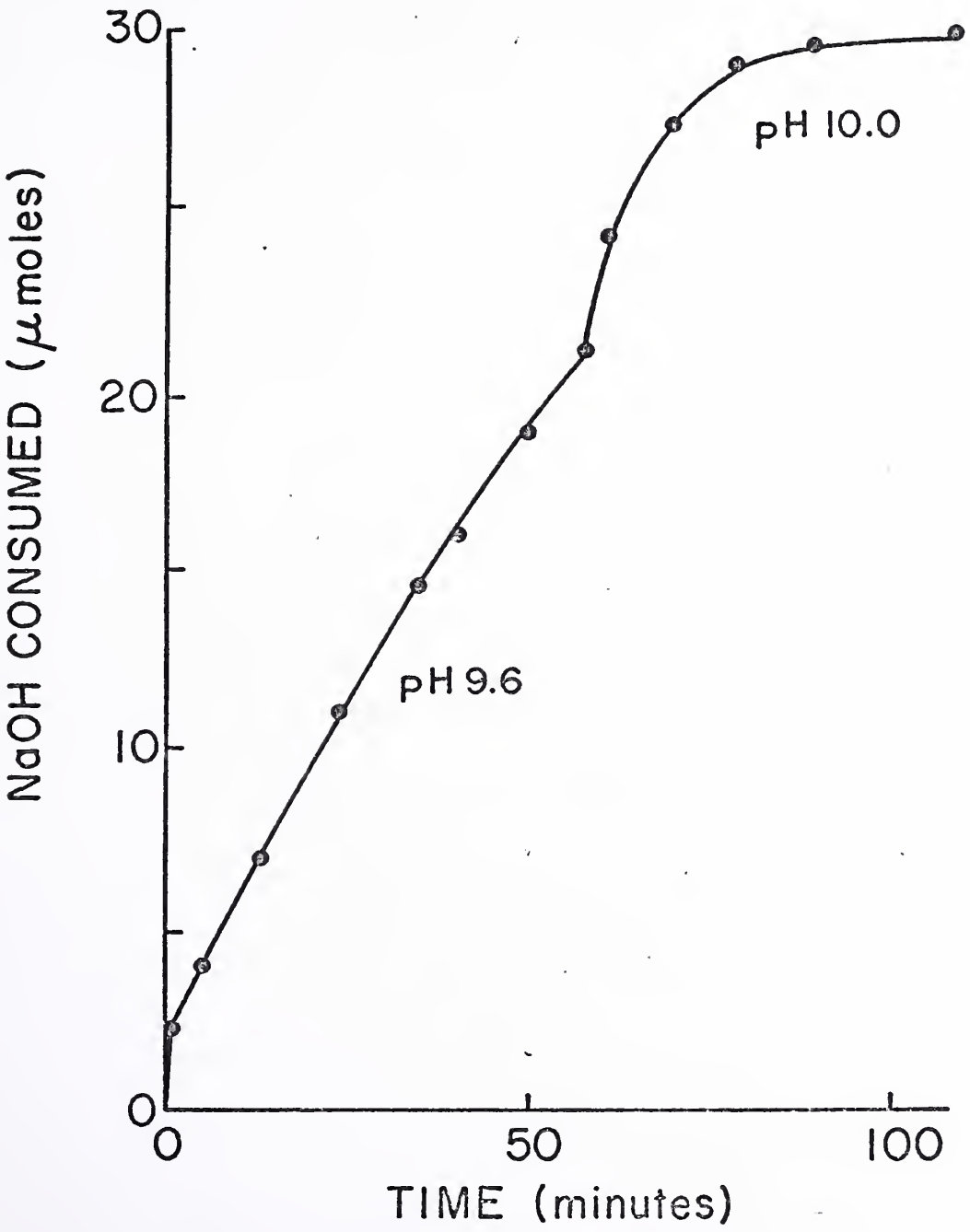


Figure 10. Consumption of Base by NAAM at pH 9.6 and 10.0. NAAM was present at an initial concentration of 36 mM (36 μmoles in 1.0 ml). At 60 minutes, the pH was readjusted to pH 10.0. Details are given in the text.

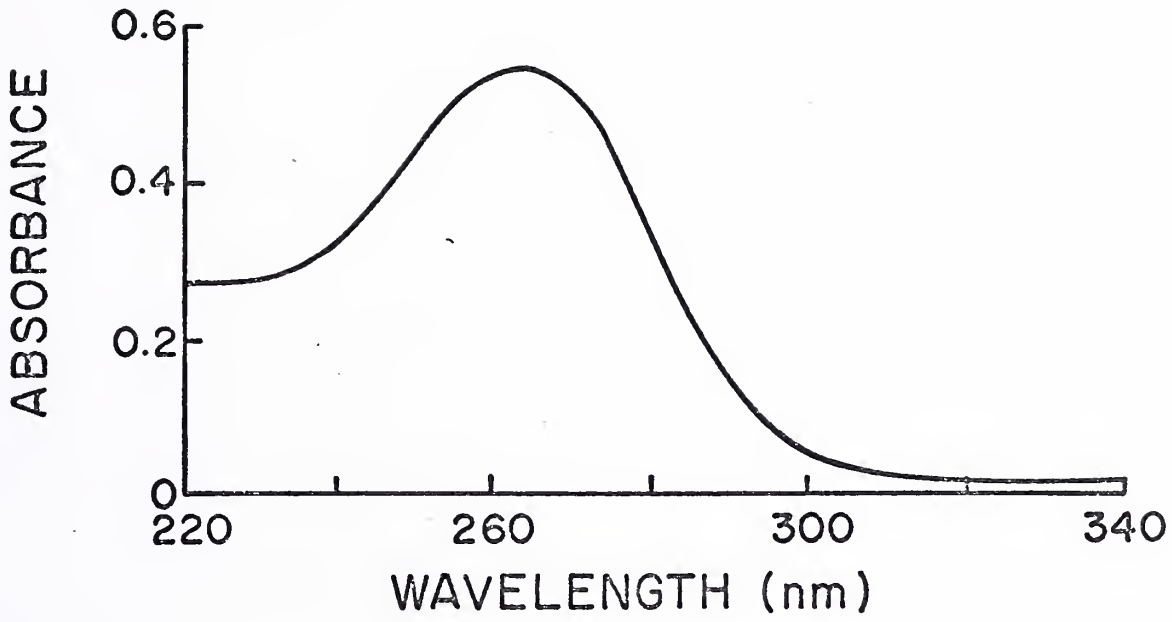


Figure 11. Ultraviolet Spectrum of Material Formed from NAAM at pH 10.0. The initial concentration of NAAM was 36 mM. The above spectrum represents 3 µl of the completed reaction mixture in 2.5 ml distilled water.

of NAAM) which suggests that formation of a product with an absorption maximum in the region of 265 nm proceeds through formation of NAAM. The product was readjusted to neutral pH and submitted to thin-layer chromatography with the solvent n-BuOH:AcOH:H_2O (4:1:1). The compound was UV absorbing, non-fluorescent, and had an R_f value of 0.57.

In an attempt to characterize further this 265 nm absorbing product, a solution of NAAM that had been converted to 265 nm absorbing material at pH 10.0 as described above was re-titrated from pH 10.0 to pH 3.1 with 0.1 N HCl (Figure 12). Although the 265 nm absorbing material had no ionizing groups in the pH range 10 to 4, an acid ionization may exist with a pK_a below 3.5.

In addition to the 265 nm absorbing material, the UV spectrum of the filtrate after removal of NAAM (Figure 9) showed the presence of another material absorbing in the 240 to 250 nm range. This was verified by thin-layer chromatography of the filtrate (readjusted to neutral pH) in the previously described solvent system which yielded four products (Figure 13). One was strongly fluorescent (R_f = 0.66) and could be attributed to the small amount of NAAM soluble in the aqueous filtrate. One product (R_f = 0.53) was only faintly visible after heating, probably representing a small amount of unreacted AcFAsnMe. The other two products (R_f = 0.34 and 0.62) appeared as dark spots under the UV lamp. The substance with an R_f value of 0.62 may represent the 265 nm absorbing material formed from NAAM (R_f = 0.57) (Figure 13).

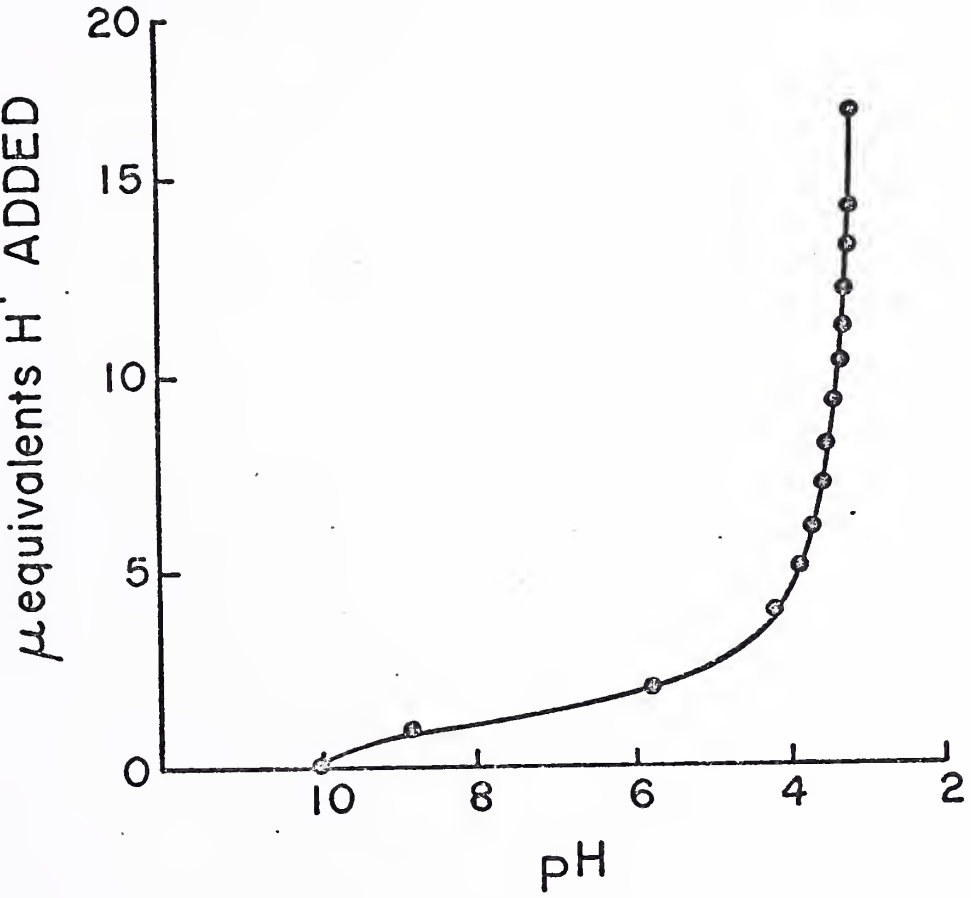


Figure 12. The Titration of 265 nm Absorbing Product from pH 10.0 to pH 3.0. Twenty-eight μmoles (23 mM, 1.2 ml) of NAAM were converted to 265 nm absorbing product at pH 10.0. Titrant = 0.1 N HCl.

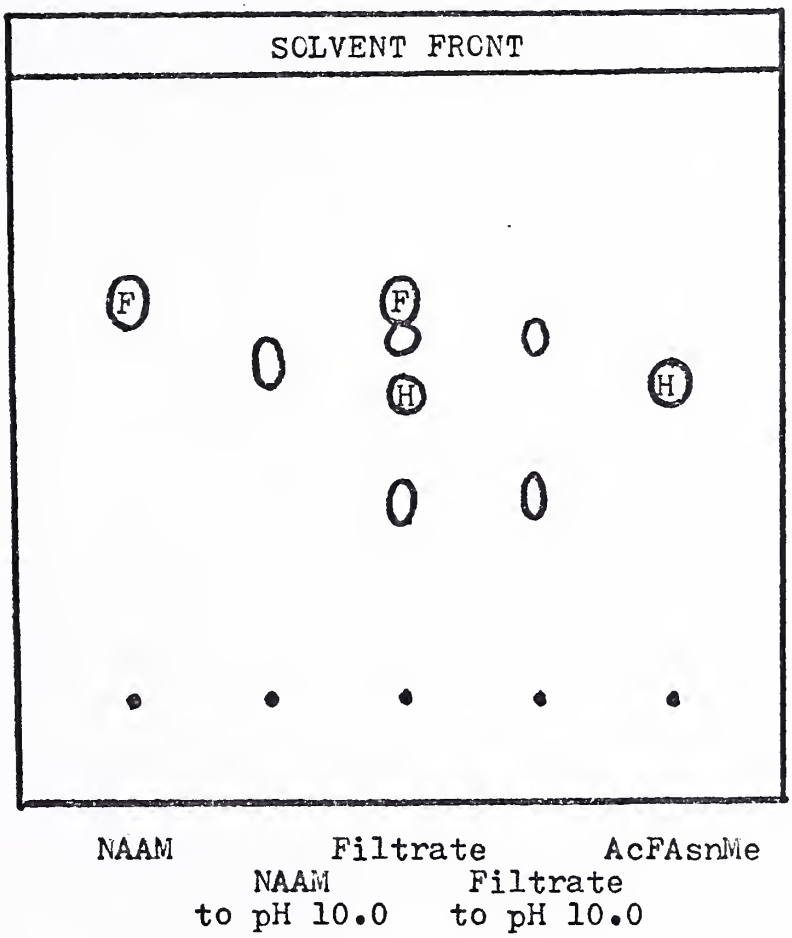


Figure 13. Thin-layer Chromatography of AcFAsnMe, NAAM, and Additional Degradation Products. The solvent contained n-BuOH:AcOH:H_2O (4:1:1). Ⓕ indicates compounds which appeared strongly fluorescent. Ⓗ indicates compounds which appeared only after heating. All other compounds appeared as dark spots under the UV lamp. Further details are given in the text.

Apparently the product with an R_f value of 0.34 is the compound which absorbs in the 240 to 250 nm range. As an additional study, the filtrate from reaction at pH 8.8 was adjusted to pH 10.0 and allowed to react until the liberation of acid ceased. A UV spectrum of the reacted filtrate showed the presence of the 265 nm absorbing material along with the questionable presence of another material absorbing in the 240 to 250 nm range (Figure 14). Thin-layer chromatography of the reacted filtrate in the above solvent revealed the presence of two UV absorbing, non-fluorescent compounds with R_f values of 0.34 and 0.62 (Figure 13). These values are identical to the R_f values of the two substances (other than NAAM and AcFAsnMe) present in the unreacted filtrate. This suggests that the product that absorbs in the 240 to 250 nm range is not converted to the 265 nm absorbing product. Since it was subsequently shown that the latter two products present in the filtrate did not inhibit leukemic cells in culture, further characterization of these compounds was not undertaken.

IV. Cytotoxicity of the Products of AcFAsnMe Degradation.

An aqueous solution of recrystallized NAAM was tested in cell culture at concentrations between 2.0×10^{-5} M and 1.6×10^{-4} M using the same methods previously described (Figure 15). It can be seen that NAAM caused an essentially identical pattern of growth inhibition when compared to AcFAsnMe. To determine if the material formed by treatment of AcFAsnMe with strong base was also cytotoxic, an aqueous

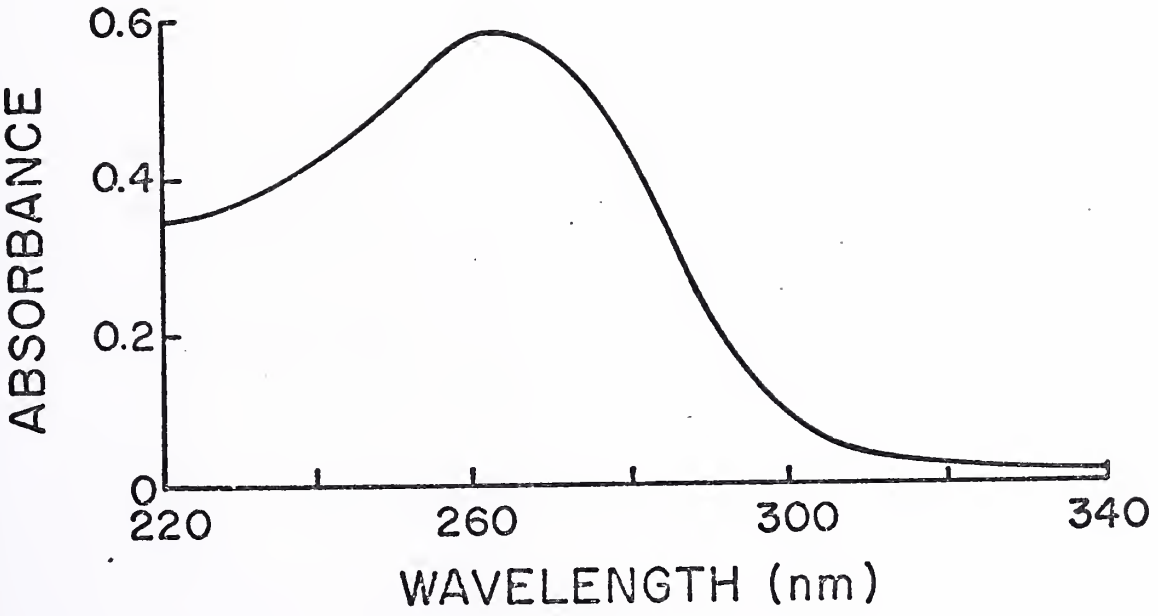


Figure 14. Ultraviolet Spectrum of Material Formed from the Filtrate. The initial concentration of AcFAsnMe was 0.36 M. The filtrate was adjusted to pH 10.0 and allowed to react until the liberation of acid ceased. The above spectrum represents 3 µl of this reaction mixture in 2.5 ml of distilled water.

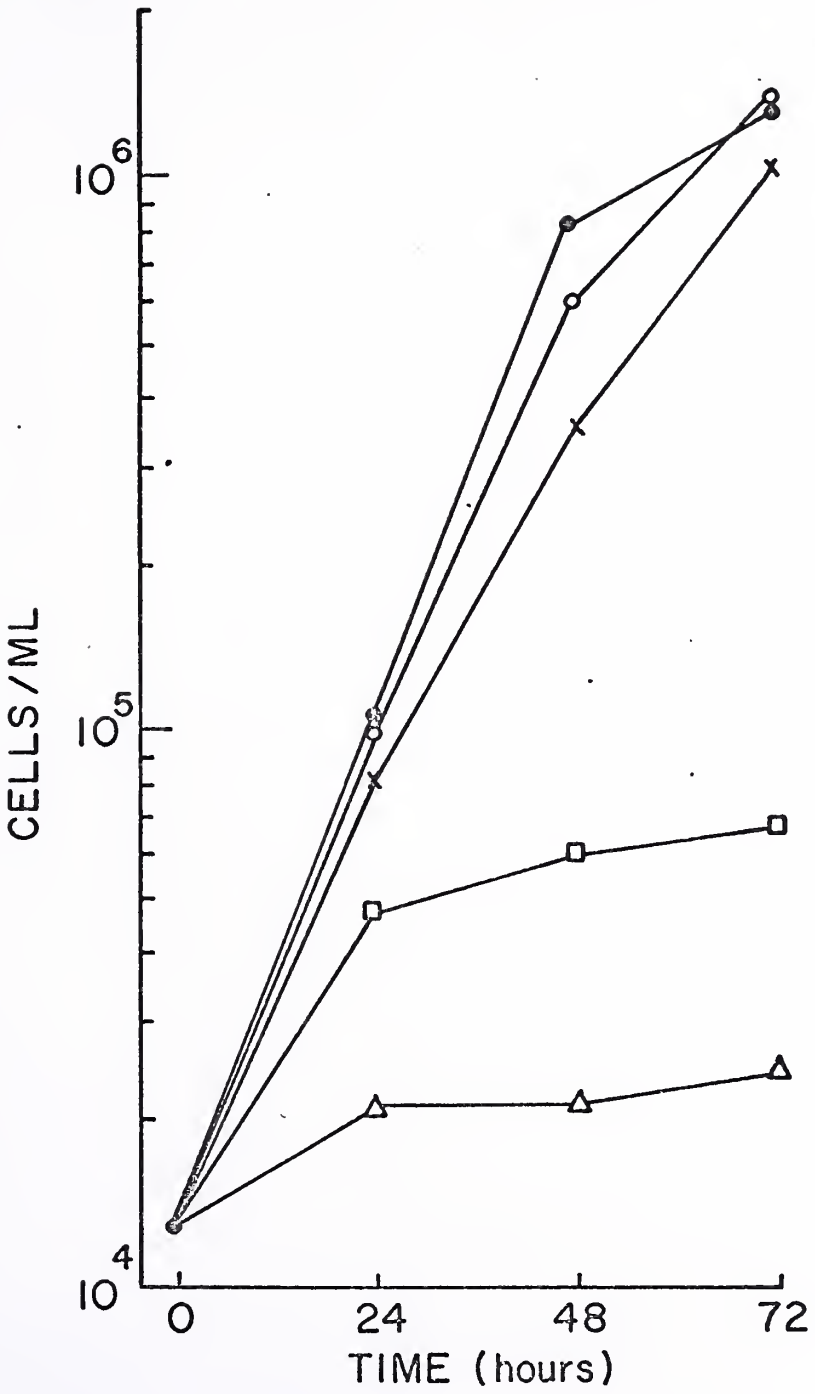


Figure 15. The Growth Inhibition of L5178Y by NAAM.

● — ● = Control
○ — ○ = 2.0×10^{-5} M NAAM
X — X = 4.0×10^{-5} M NAAM
□ — □ = 8.0×10^{-5} M NAAM
△ — △ = 1.6×10^{-4} M NAAM

solution of AcFAsnMe was titrated to pH 12 and allowed to react until the liberation of acid ceased. The resulting UV spectrum was identical to that seen in Figure 14. The solution was readjusted to neutral pH, sterile filtered, and tested in cell culture by the methods previously described at concentrations between 6.1×10^{-5} M and 4.8×10^{-4} M based on the original concentration of AcFAsnMe. It was evident that this material was not contributing to inhibition of cell growth (Figure 16). In addition, a 100-fold dilution of the filtrate after removal of NAAM, which would be the equivalent of 1.1×10^{-4} M AcFAsnMe, was also tested in cell culture and found to be inactive (Figure 17). Thus, any other reaction products from AcFAsnMe formed in the culture (or at pH 8.8) were essentially inactive as inhibitors of the growth of the lymphoblasts.

To determine whether the fluoride ion liberated from AcFAsnMe in the form of HF during incubation had any effect on cell growth, NaF at concentrations between 2.4×10^{-4} and 6.1×10^{-5} M was tested in cell culture (Figure 18). No significant inhibition of cell growth was seen which is in agreement with the data of Duschinsky (22).

It is therefore concluded that the growth inhibition of L5178Y by AcFAsnMe can be attributed to formation of the acetyl-aminomaleimide structure.

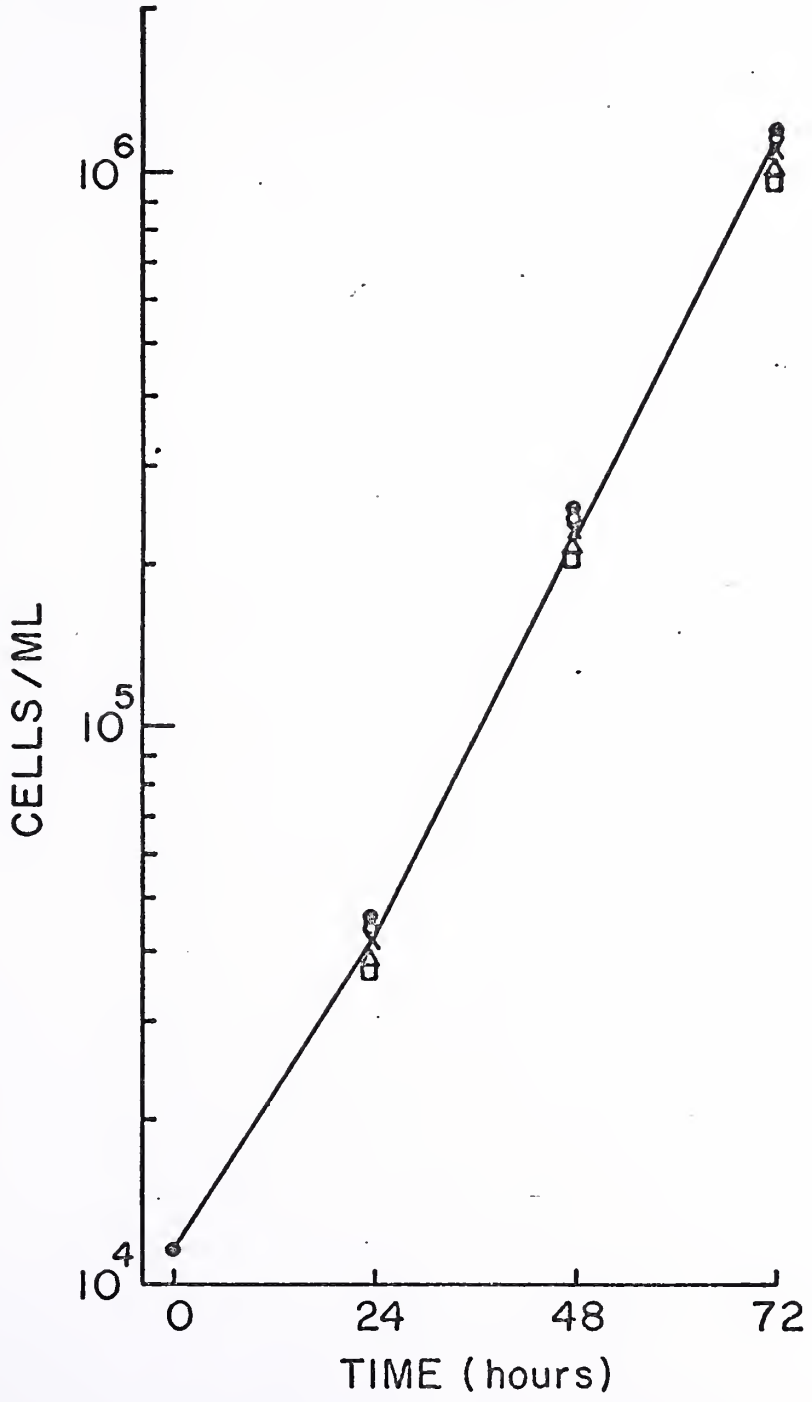


Figure 16. The Effect of Material Formed from Reaction of AcFAsnMe at pH 12.0. The concentrations below are based on the initial concentration of AcFAsnMe (4.8×10^{-4} M).

- ●——● = Control
- ○——○ = 6.1×10^{-5} M (AcFAsnMe)
- ×——× = 1.2×10^{-4} M (AcFAsnMe)
- △——△ = 2.4×10^{-4} M (AcFAsnMe)
- □——□ = 4.8×10^{-4} M (AcFAsnMe)

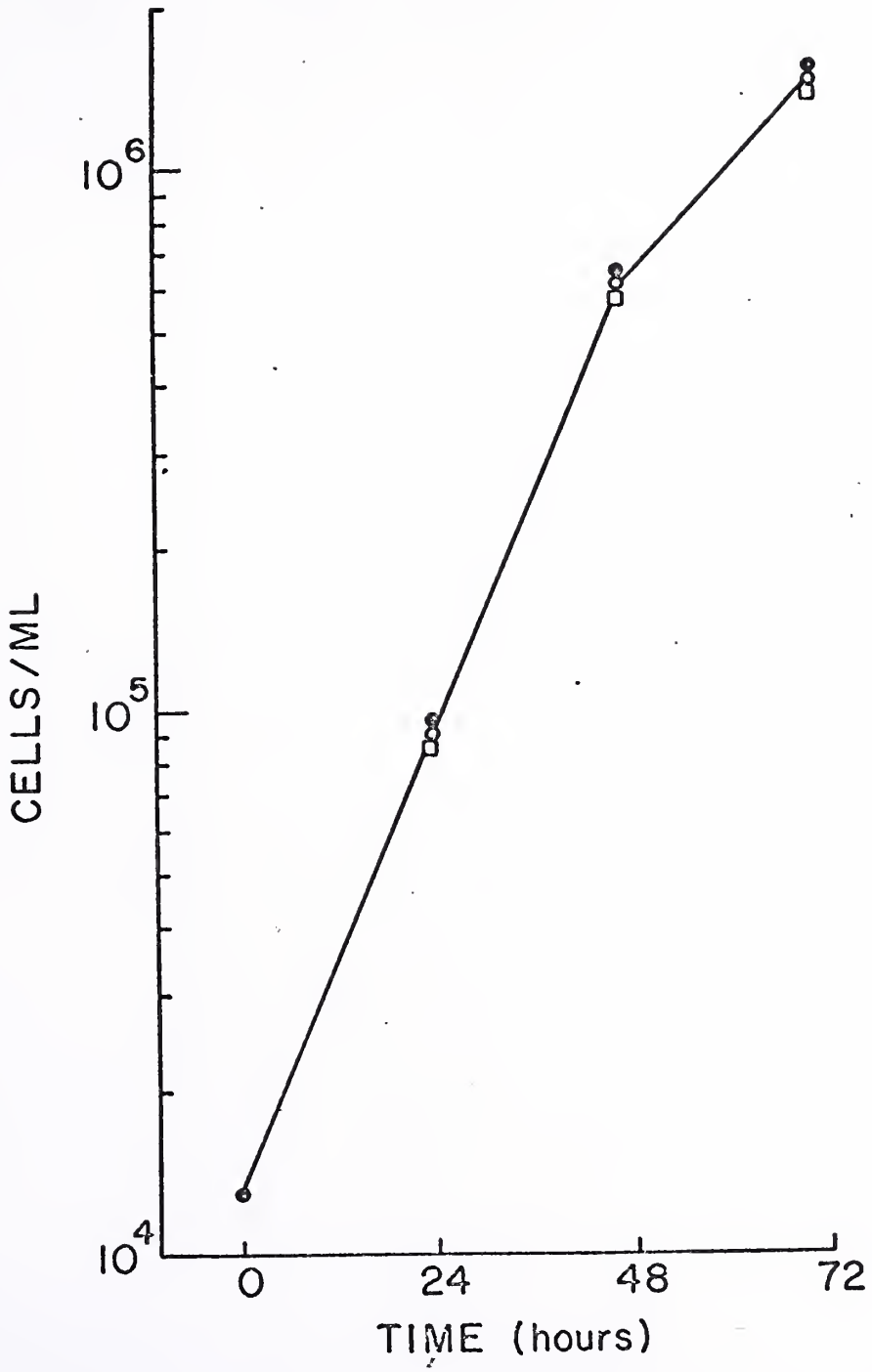


Figure 17. The Effect of Filtrate on the Growth of L5178Y Cells. The initial concentration of AcFAsnMe was 0.15 M. After completion of the reaction at pH 8.8, NAAM was removed by filtration. Further details of the experimental technique are given in the text. The concentrations below are based on the initial concentration of AcFAsnMe.

●——● = Control
○——○ = 5.0×10^{-5} M (AcFAsnMe)
□——□ = 1.1×10^{-4} M (AcFAsnMe)

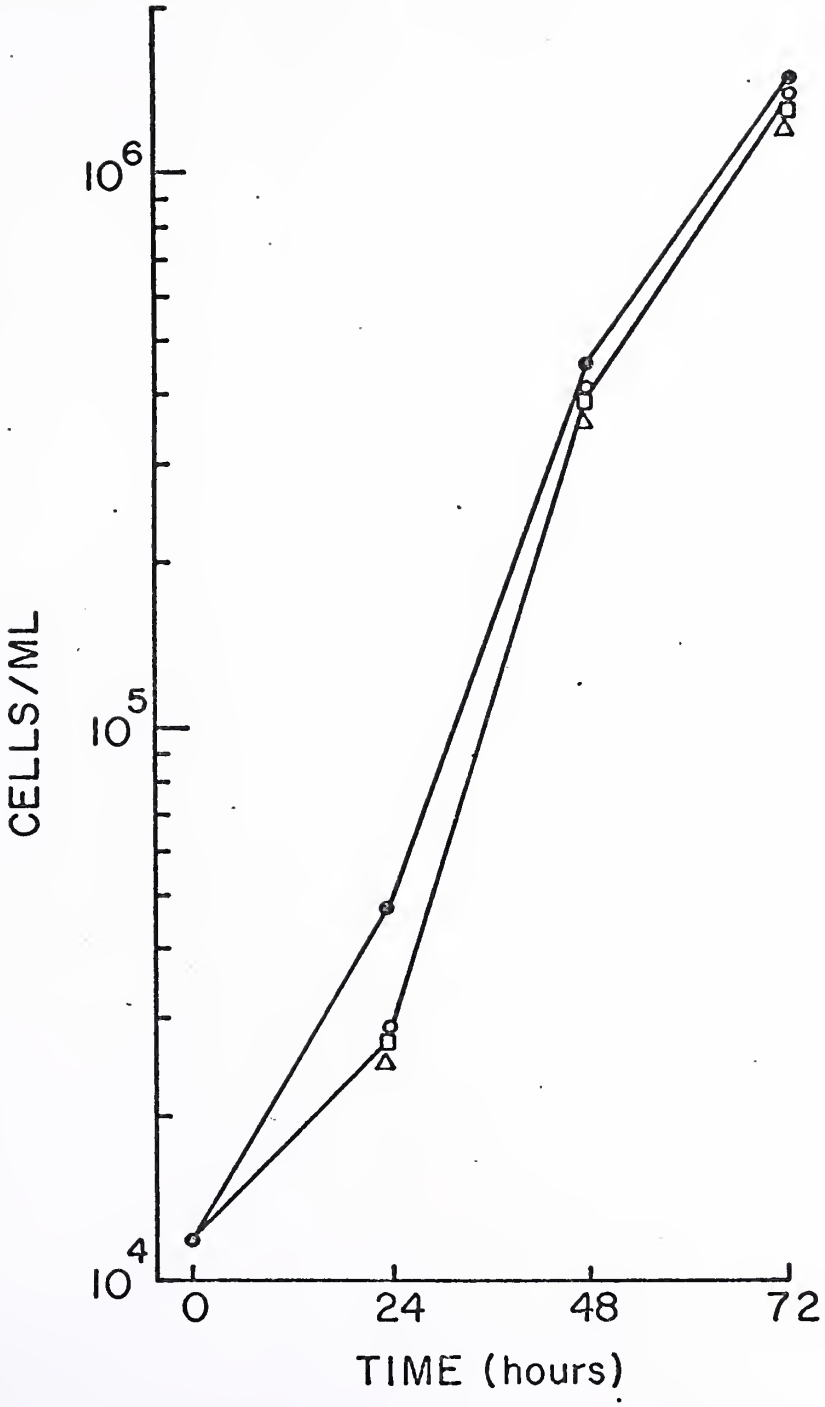


Figure 18. The Effect of NaF on the Growth of L5178Y Cells.

● — ● = Control
○ — ○ = 6.1×10^{-5} M NaF
□ — □ = 1.2×10^{-4} M NaF
△ — △ = 2.4×10^{-4} M NaF

DISCUSSION

DISCUSSION

The initial studies with cell culture in this thesis resulted in the introduction of 24-well Linbro plates for testing the cytotoxicity of drugs. This system makes possible the growth of cells in only 1 ml of media which is particularly useful when only small quantities of drug are available. In addition, several conditions can be included on one plate affording simple and rapid set up of assays and harvesting for the determination of cell counts. Contamination never occurred during the 72 hour assays and L5178Y cells grew at doubling rates as low as 9 to 10 hours which are as good if not better than those found using standard cell culture tubes with 5 ml of medium (30).

The intended theme of this research was that D,L-threo-N^2-acetyl-3-fluoroasparagine methyl ester (AcFAsnMe) might function as an analog of asparagine. Although this compound was shown to be cytotoxic for cell cultures at concentrations reported by Duschinsky, the proposed mechanism of action must now be questioned. Metabolic liberation of free 3-fluoroasparagine was expected to either limit the availability of L-asparagine to the cell, be incorporated in its place, or feedback inhibit asparagine biosynthesis. Such a mechanism might have been hoped to expand the spectrum of L-asparaginase therapy. This mode of action became less likely since addition of large amounts of asparagine to cell cultures could

only minimally prevent the cytotoxic effects of AcFAsnMe. Furthermore, glutamine was approximately equal in its effect, suggesting that this was not an asparagine specific effect. In fact, control cultures supplemented with excesses of these amino acids grew slightly better. This could account for the apparent increase in growth seen when these amino acid supplements were added to cultures containing AcFAsnMe. In addition, Dr. David Cooney at the National Institutes of Health tested AcFAsnMe as a possible inhibitor of asparagine synthetase. At a final concentration of 1 mM, no inhibition of crude asparagine synthetase derived from tumor or pancreatic tissue was seen (31). These experiments of course did not exclude potential activity of the unblocked analog. Finally, as shown below, the equal activity of 2-(N-acetyl)-aminomaleimide (NAAM) as a cytotoxic agent would suggest that all cytotoxic activity of AcFAsnMe is actually a consequence of the chemical degradation product formed in tissue culture medium in the absence of leukemic cells.

Prior to this finding, however, a number of attempts were made to de-block AcFAsnMe with esterases. These were unsuccessful probably because of steric hindrance by the N-acetyl group which blocked entry of AcFAsnMe into the active site. The inability of the tri-fluoro-N-acetyl methyl ester to act as a substrate supports this idea. The activity of pseudocholinesterase, in particular, declines rapidly as substrates lose their structural resemblance to butyrylcholine (32), and chain branching in the carbon atom next to the ester link

has a particularly adverse effect on the rate of hydrolysis (33). Pseudocholinesterase is known to hydrolyze non-choline esters, but a structural resemblance to butyrate is preferable (34). On the other hand, the purified carboxylesterases tested in these experiments could have been expected to hydrolyze AcFAsnMe at a significant rate, as these enzymes possess a wide range of specificity (35). Carboxylesterases in general are widely distributed in vertebrate tissues and blood serum, with the highest activities in mammals being in the liver, kidneys, duodenum and brain (35). However, even when a 20% by volume supernatant (100,000g) from mouse liver homogenate was added to AcFAsnMe, a relatively low rate of hydrolysis resulted. For this reason, along with increasing evidence that AcFAsnMe was not acting as an asparagine analog, further studies such as purification of the esterases present in the homogenate were not undertaken.

Similarly, several attempts at deacylation of AcFAsnMe by hog kidney acylase were also unsuccessful. In this case, however, purified acylases other than that from hog kidney were not available for testing. Here, also, increasing evidence that AcFAsnMe itself was not the compound responsible for inhibition did not encourage further attempts at deacylation.

Therefore, the main focus of this thesis turned from AcFAsnMe to the characterization of the degradation products formed during incubation of AcFAsnMe in media at 37°. At least one of these products was responsible for inhibition

of L5178Y cells based on the results of the pre-incubation studies. It was indeed fortunate that during the reaction of relatively high concentrations of AcFAsnMe at pH 8.8, one of the products was only sparingly soluble in water and therefore precipitated. Thus, further characterization of this product was possible.

Proton and fluorine magnetic resonance spectra, mass spectroscopy studies, and elemental analysis were all consistent with the loss of HF and MeOH to form NAAM. An ultraviolet spectrum verified that this was the material responsible for absorption at 233 nm. It might be noted that showdomycin, a nucleoside antibiotic with a maleimide structure, has an ultraviolet maximum at 220 nm (36). The property of fluorescence which an aqueous solution of NAAM possesses is also consistent with the known fluorescence of certain maleimide derivatives (37). The results from the titration of NAAM also reflect its maleimide structure. During rapid titration of NAAM from pH 6.5 to pH 9.5 prior to formation of 265 nm absorbing material, less than 2 μmoles of base were required with 36 μmoles of NAAM available for titration. This strongly suggests that there are no ionizing groups in this pH range. Similarly, during titration of NAAM from pH 5.5 with acid, there were no titratable groups at least down to pH 3. The pK_a of the imide proton in maleimide is 9.5 (36), but it appears that the pK_a of the imide proton in NAAM is above 9.5.

Crystalline NAAM caused an essentially equal degree of growth inhibition when compared to AcFAsnMe. This is in agreement with the studies involving pre-incubation of AcFAsnMe in media prior to addition of cells. Since the other degradation products formed during incubation of AcFAsnMe were shown to be inactive in cell culture, NAAM was concluded to be the compound responsible for inhibition of L5178Y cells. Presumably, this previously unreported compound acts as an alkylating agent as numerous other maleimide derivatives have been shown to function. N-Ethylmaleimide is known to react rapidly and specifically with sulfhydryl groups (38,39). Showdomycin selectively inhibits certain enzymes, probably due to the alkylating property of its maleimide structure (40). More specifically, maleimide itself has recently been shown to be a potent and irreversible inhibitor of the L-asparagine synthetase from L5178Y asparagine-independent mouse leukemia (41). L-Asparagine did not reduce the lethal effects of maleimide <u>in vivo</u>, thus eliminating the possibility of its acting as a substrate analog. It was concluded from this study that maleimide inhibited the enzymes of L-asparagine metabolism due to its ability to form covalent bonds with critical sulfhydryl compounds (41). In this regard, some initial studies with NAAM indicated that it did react with L-cysteine, a strong nucleophile. Further studies in this area would be needed to confirm the action of NAAM as an alkylating agent. These could include reaction of NAAM with other strong nucleophiles such

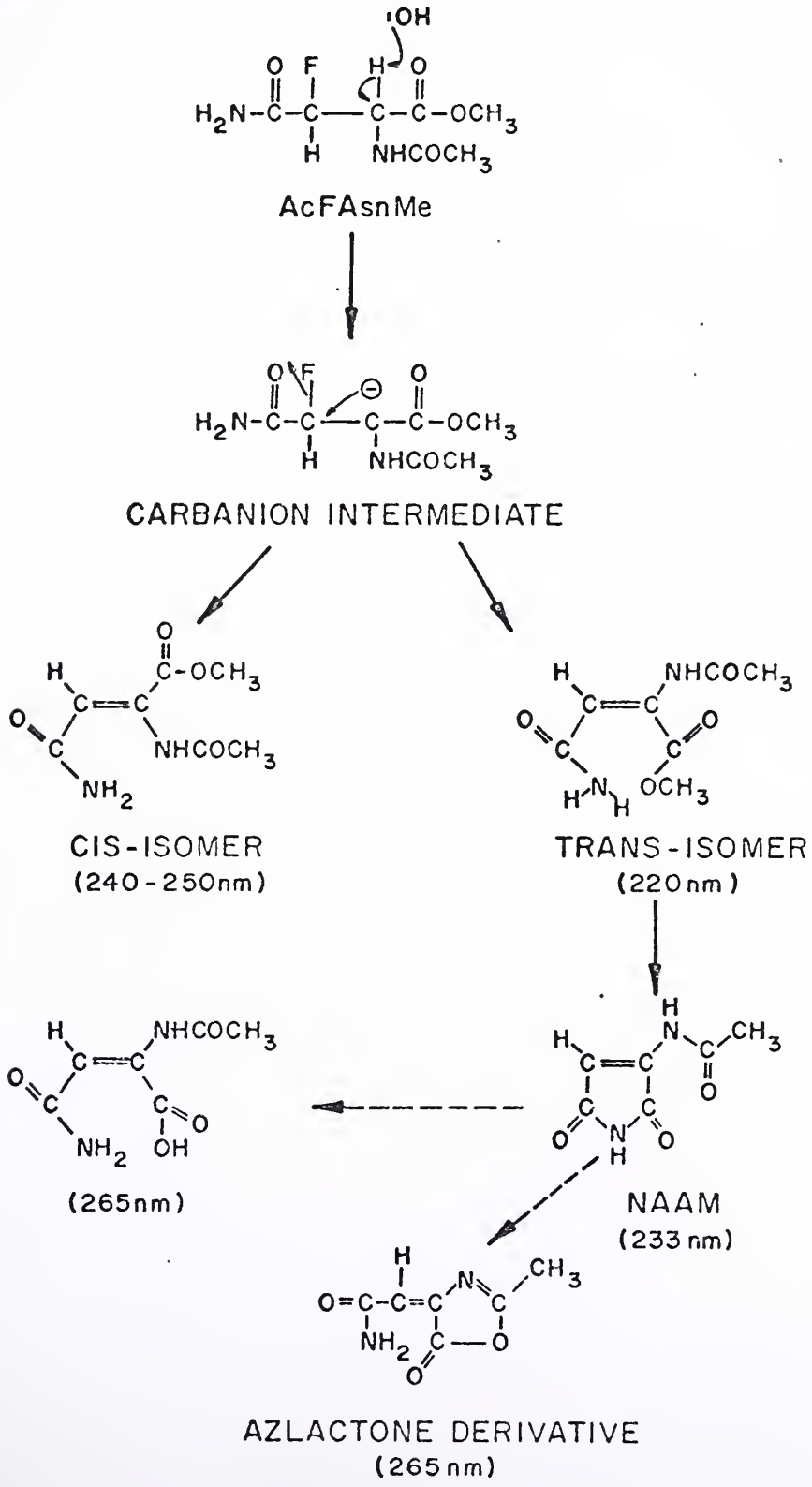
as glutathione, which is known to react with maleimides (38). Alternatively, glutathione could be incubated with NAAM under culture conditions to determine whether the degree of growth inhibition could be nullified, presumably by binding of glutathione to NAAM to inactivate the latter. In fact, maleimides are known to react with amino acids (39) and perhaps this could explain, at least in part, why additional quantities of asparagine or glutamine in cultures with NAAM showed an increase in growth rate when compared with cultures that did not contain the amino acid. In addition, however, the increased growth rate in control cultures must be acknowledged to be responsible for the apparent reduction in inhibition.

The results from incubation of NAAM with L5178Y cells also indicate that the inhibition of growth is time-dependent, with the degree of inhibition becoming greater after 24 hours of incubation (see Figure 15, Results). A similar effect was seen with maleimide which was unable to inhibit L-asparagine synthetase in vivo soon after injection (41). The time course of the inhibition by NAAM suggests that the compound is inhibiting protein synthesis. This possibility could be further investigated with in vivo studies to determine the effect of administration of NAAM on the incorporation of a radioactively labeled amino acid into the protein of L5178Y cells. Alternatively, NAAM may have to be metabolized before it can act as an inhibitor of certain enzymes, which could be determined by following the fate of radioactively labeled NAAM.

Although the biological properties of NAAM described above were important aspects of this thesis, the chemistry of the formation of this compound as well as the other products formed during incubation of AcFAsnMe was a main focus. From the results of these chemical studies, the formation of NAAM under physiologic conditions and at pH 8.8 was proposed to occur according to the mechanism shown in Figure 19. Although detailed kinetic studies were not performed during the course of these experiments, it did appear that there was a five-fold increase in the rate of acid liberation when the pH was increased from 8.8 to 10.0. This suggests that the initial reaction is a general base-catalyzed reaction. The reaction of base with AcFAsnMe might be expected to involve α-proton abstraction with formation of a carbanion as depicted in Figure 19. This would readily lead to β-elimination of the fluorine atom (42). An analogous situation is the interaction of base on the surface of enzyme bound to pyridoxal phosphate with compounds such as β-fluoro-D-L-alanine to effect elimination of HF (43). The formation of the carbanion is necessary to explain the lack of a quantitative conversion of AcFAsnMe to NAAM. Although AcFAsnMe is in the threo- configuration, if it were to liberate HF through this planar intermediate, both cis- and trans- isomeric products would result. Presumably, the trans- isomer was then free to ring close with loss of MeOH to form a maleimide which precipitated due to its relative insolubility in water. This trans- isomer may be the material with UV absorption in the

FIGURE 19

THE PROPOSED REACTION SCHEME FOR AcFAsnMe



220 nm range which precedes formation of the 233 nm material (see Figure 7, Results). It is assumed that the other isomer was water soluble and remained in the filtrate. This was confirmed by ultraviolet spectroscopy and thin-layer chromatography. It appears that this cis- isomer is the material responsible for absorption in the 240 to 250 nm range. Results also indicated that this compound was not converted to the 265 nm absorbing product upon base addition to the filtrate and that it remained unchanged. If one assumes that one-half of the starting material was converted to each of the isomers, as it appears from the quantititive yield of NAAM from AcFAsnMe, the cis- isomer was found to be inactive against L5178Y cells at a calculated concentration of 5×10^{-5} M.

According to the mechanism depicted in Figure 19, the liberation of acid from AcFAsnMe in the form HF would be expected to correspond to formation of both the cis- and trans- isomers rather than to formation of NAAM with liberation of MeOH. This was tested by plotting the increase in absorbance at both 220 nm (putative trans- isomer) and 233 nm (NAAM) during the previously described reaction of AcFAsnMe (10^{-4} M) in 0.05 M borate buffer pH 8.8. These curves were then compared to the liberation of acid during reaction of AcFAsnMe (0.01 M) in distilled water (Figure 20). The shape of the latter curve appears to correspond more to the shape of the curve showing change in absorbance at 220 nm, which suggests that the liberation of acid does correlate with the formation

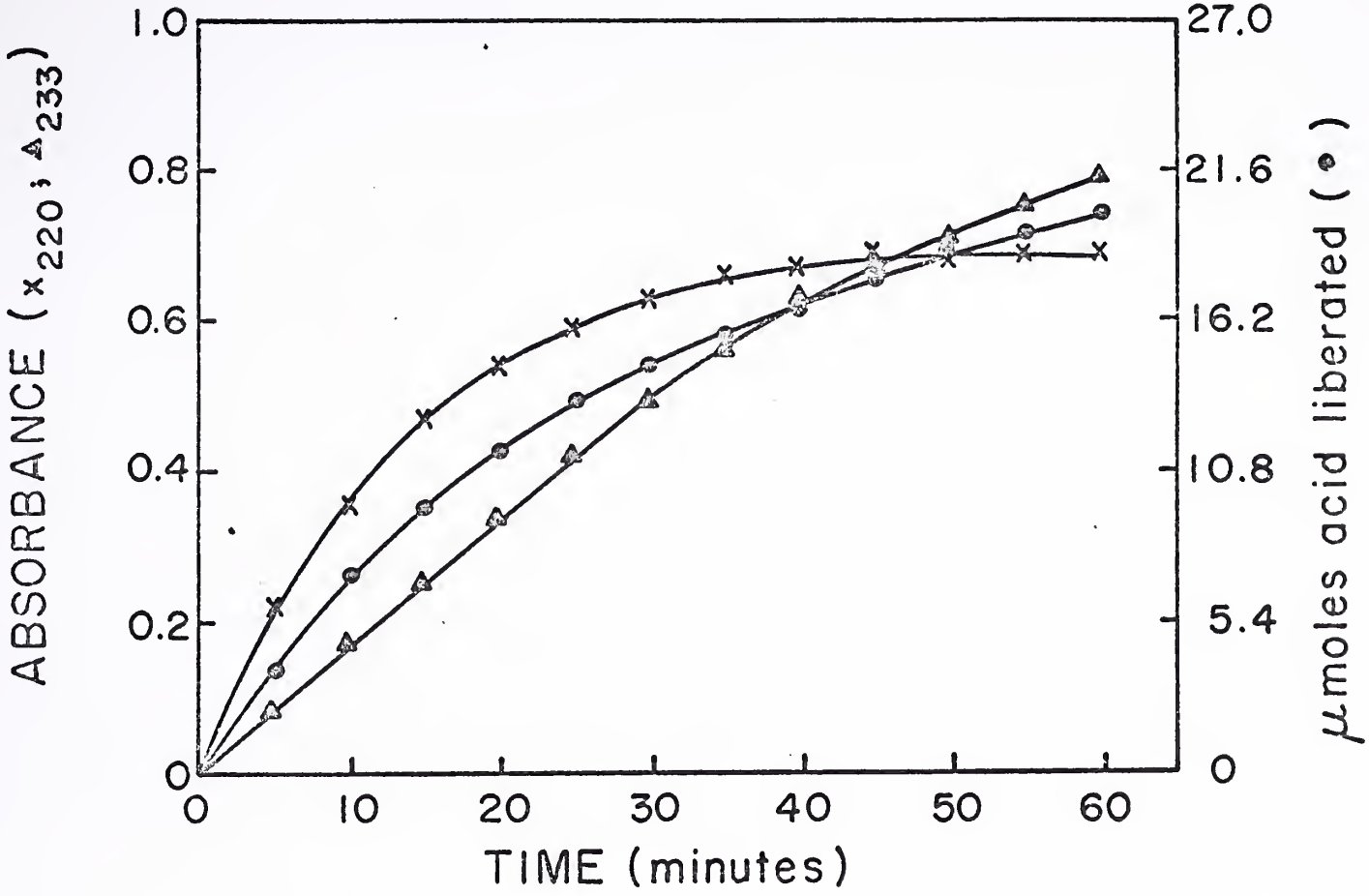


Figure 20. The Correlation of the Liberation of Acid with the Increase in Absorbance at 220 nm During the Reaction of AcFAsnMe at pH 8.8. Details are given in the text.

of the trans- compound. However, more detailed kinetic studies would be needed to confirm this.

The structure of the 265 nm absorbing product is less certain. Possibilities include removal of the N-acetyl amide proton by base with formation of an azlactone derivative (Figure 19). The high degree of unsaturation and the existence of other resonance structures for this compound might explain its ability to absorb at higher wavelengths. However, azlactones are routinely prepared by the reaction of α-acylamido acids with sodium acetate and acetic anhydride at 100° (44), a rather severe set of conditions, whereas the formation of the 265 nm absorbing product occurs to some degree at pH 8.8, 25°. Another more likely possibility for formation of the 265 nm absorbing product is that base addition results in removal of the -NH proton of the maleimide ring which is then followed by base hydrolysis to give ring opening. As examples, both maleimide and showdomycin have been shown to undergo such a reaction upon exposure to base. In fact, the UV maximum obtained in water for these compounds (~275 nm) shifts to higher wavelengths (~325 nm) upon exposure to base, which may be analogous to the shift from 233 to 265 nm upon base addition to NAAM. The formation of this 265 nm absorbing material could explain the addition of approximately one mole of base for each mole of NAAM present, being equivalent to titration of the -NH proton. This new compound would also be expected to contain an ionizing group, presumably a carboxyl group, in the pH 3 range,

as was suggested during titration of the compound. However, ring opening would also make an $-NH_2$ group available for titration and this was not apparent. Clearly, further characterization of this compound would be necessary in order to determine its actual structure.

In summary, this thesis investigated D.L-threo-N^2-acetyl-3-fluoroasparagine methyl ester (AcFAsnMe) as a cytotoxic agent against L5178Y leukemic cells. It was found that this drug did not act as an asparagine analog as was initially assumed, but rather through formation of 2-(N-acetyl)-aminomaleimide (NAAM), which presumably acts as an alkylating agent. This product, a previously unreported chemical entity, was characterized and a mechanism for its formation proposed. In addition, the biological and chemical properties of the other degradation products formed on incubation of AcFAsnMe in media were studied. Finally, the question might be raised as to whether NAAM would have any potential application in the field of cancer chemotherapy. More specifically, could NAAM offer any advantages over other alkylating agents such as maleimide? In this regard, it has been shown that L-asparagine synthetase exhibits distinctive susceptibility to maleimide (41) and therefore this maleimide derivative may still be considered for use in enhancing the oncolytic effect of L-asparaginase. Further studies on the *in vivo* cytotoxicity of NAAM in animal models including the possible toxic side effects are needed in order to answer these questions.

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